Identifizierung von agonistischen und invers agonistischen Eigenschaften determinierender Strukturen in Liganden am ADP-Rezeptor $P2Y_{12}$

Dissertation
zur Erlangung des akademischen Grades
Dr. med.

an der Medizinischen Fakultät
der Universität Leipzig

eingereicht von: Philipp Schmidt
geb. 11.01.1989 in Erfurt

angefertigt an: Universität Leipzig, Medizinische Fakultät
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Beschluss über die Verleihung des Doktorgrades vom: 24.02.2016

„Eine neue wissenschaftliche Wahrheit pflegt sich nicht in der Weise durchzusetzen, dass ihre Gegner überzeugt werden und sich als belehrt erklären, sondern vielmehr dadurch, dass ihre Gegner aussterben und dass die heranwachsende Generation von vornherein mit der Wahrheit vertraut geworden ist."

(Max Planck (1948) Wissenschaftliche Selbstbiographie.
Leipzig: Johann Ambrosius Barth Verlag, S.22)

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Bibliographische Beschreibung

Schmidt, Philipp

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Universität Leipzig, Dissertation

48 S., 49 Lit., 4 Abb., 1 Publ., 4 Anl. (Suppl. Mat.)

Referat:

Die vorliegende Arbeit untersucht die strukturellen Grundlagen agonistischer und invers agonistischer Eigenschaften von Liganden am ADP-Rezeptor $P2Y_{12}$. Dazu wurde eine Bibliothek systematisch veränderter Purinverbindungen am Wildtyp-$P2Y_{12}$ (WT) mit und ohne ADP und an 28 konstitutiv aktiven $P2Y_{12}$-Mutanten getestet. Dies ermöglichte die pharmakologische Zuordnung der Substanzen als Agonist, Antagonist oder inverser Agonist. Die Untersuchungen wurden in einem Hochdurchsatz-Hefe-Expressionssystem in Hefen durchgeführt. Als agonistische Liganden am $P2Y_{12}$ Rezeptor konnten verschiedene ATP und ATP-Derivate identifiziert werden. Ihre agonistische Potenz am ADP-Rezeptor reihte sich wie folgt: 2-(methylthio)-ADP > 2-(methylthio)-ATP > ADP > ATP. In Dockingstudien wurde mittels eines komparativen Computermodels des $P2Y_{12}$ für diese ATP-Derivate eine Bindungsstelle nachgewiesen, die von den Transmembrandomänen (TM) 3, 5, 6 und 7 gebildet wird. Die

Aminosäuren Y105, E188, R256, Y259 und K280 besitzen in der Ligandeninteraktion einen besonderen Stellenwert.

Zudem konnten einige Liganden identifiziert werden, die invers agonistische Eigenschaften an konstitutiv aktiven $P2Y_{12}$-Mutanten zeigten. So führte eine N-Methyl-anthraniloyl-(mant) Modifizierung an der 3'-OH Gruppe der 2'-Deoxyribose (mant-dATP, mant-dADP) zu Liganden mit invers agonistischen Eigenschaften an 10 konstitutiv aktiven $P2Y_{12}$-Mutanten. Diese Wirkung konnte mittels verschiedener funktioneller Tests in Säugerzellsystemen ebenfalls für den WT-Rezeptor bestätigt werden. Basierend auf den Ergebnissen computerassistierter Dockingstudien schienen inverse Agonisten und Agonisten dieselbe Bindungstasche zu nutzen. Eine mechanistische Erklärung für ihren funktionellen Unterschied am WT konnte das Modell jedoch nicht liefern.

Zusammenfassend wurde gezeigt, dass der als ADP-Rezeptor bezeichnete $P2Y_{12}$ mit einer etwas geringeren Potenz auch ATP als natürlichen Agonist erkennt und dass mant-modifiziertes dATP und dADP neue inverse Agonisten mit potenziellem therapeutischem Nutzen sind.

Einleitung

Definition und Klassifizierung der G-Protein-gekoppelten Rezeptoren

Ein G-Protein-gekoppelter Rezeptor (GPCR) ist ein transmembranäres Protein, welches äußere Signale (extrazellulär) in die Zelle leitet (intrazellulär) und dort über GTP-bindende Proteine Signalprozesse auslöst. GPCRs sind die größte und vielfältigste Gruppe unter den Rezeptorproteinen. Im menschlichen Genom kodieren ca. 900 Gene für GPCRs (Fredriksson and Schioth, 2005), wovon 50% den olfaktorischen Rezeptoren zugeordnet werden. In der Vergangenheit wurden sie bei Vertebraten in drei Klassen unterteilt:

Klasse A: Die rhodopsinähnlichen Rezeptoren
Klasse B: Die Sekretinrezeptoren
Klasse C: Die metabotropischen Glutamatrezeptoren

Die rhodopsinähnlichen Rezeptoren bilden mit über 85% die größte Rezeptorfamilie unter den GPCRs. Neben den drei Vertebraten-Klassen werden zusätzlich die Klassen D und E unterschieden. Hierbei handelt es sich um Pheromonrezeptoren in Hefen und cAMP-Rezeptoren in Fadenwürmern.
Eine neuere, auf phylogenetischen Eigenschaften basierende, Einteilung der GPCRs nach dem GRAFS-/Fredriksson-System erfolgt in die folgenden 5 Gruppen: Glutamat-, Rhodopsin- , Adhäsions-, Frizzled/Taste2- und Sekretinrezeptoren (Fredriksson et al., 2003). Ihre Signaltransduktion geschieht über GTP-bindende Proteine (G-Proteine). Diese heterotrimeren Proteine besitzen in der α-Untereinheit eine GTP/GDP Bindungsdomäne (Gilman, 1987; Wess, 1998) und unterscheiden sich in drei wesentlichen Formen :

1. Die Stimulation ($G\alpha_s$–Protein) oder Hemmung ($G\alpha_{i/o}$–Protein) der Adenylylcyclase führt zu einer Änderung des cAMP-Spiegels.
2. Die Stimulation der Phospholipase C ($G\alpha_q$–Protein) führt zur Bildung von „second messengern" Diacylglycerol (DAG) und Inositol-1,4,5-triphosphat (IP3) aus Phosphatidyl-inositol-4,5-diphosphat (PIP2).
3. Kleine GTP bindende Proteine werden durch die Aktivierung von Gα12/13–Proteinen beeinflusst (Wettschureck and Offermanns, 2005).

Darüber hinaus können auch die β-/γ-Untereinheiten selbst über Effektoren wie z.B. Ionenkanäle oder Enzyme an der Signaltransduktion beteilig sein (Wu et al., 1998). Aufgrund ihrer einheitlichen Struktur aus sieben Transmembrandomänen (TM), werden die GPCRs auch 7TM-Rezeptoren genannt. Die Vielseitigkeit der GPCRs zeigt sich zudem in der Agonistenvielfalt. Neurotransmitter, Hormone, Nukleotide, Geschmacks- und Geruchsstoffe aber auch physikalische Reize, wie Licht, werden transformiert. So reichhaltig wie ihre physiologische Relevanz ist auch die pharmakotherapeutische Bedeutung der GPCRs. Bekannte und medizinisch unverzichtbare Wirkstoffe, wie beispielsweise Betablocker, Opioide, Thrombozytenaggregationshemmer und Neuroleptika, haben u.a. ihren Angriffspunkt an GPCRs. In der heutigen Praxis wirken ca. 40 % aller modernen Pharmaka an diesen Rezeptoren (Overington et al., 2006).
Da GPCRs an fast allen physiologischen Vorgängen im Körper beteiligt sind, ist es naheliegend, dass ein direkter oder indirekter Defekt dieser zu diversen Krankheiten führt. Mutationen in GPCR-Genen verursachen unterschiedlichste Erkrankungen, wie Seh- und Wachstumsstörungen oder die Entstehung maligner Tumoren (Schöneberg et al., 2004).

Purinerge Rezeptoren

Man unterscheidet zwei Familien von purinergen Rezeptoren. P1-Rezeptoren werden durch das Purinnukeosid Adenosin und P2-Rezeptoren durch Nukleotide, meist durch ATP, aktiviert (Burnstock G, 1980). Die Adenosin-Rezeptoren werden ihren molekularen, biochemischen und pharmakologischen Eigenschaften nach in vier verschiedene Subtypen unterteilt:

A1-, A2a-, A2b- und A3-Rezeptoren.

Die P2-Rezeptoren untergliedern sich weiterhin in die P2X-Rezeptoren, welche in der Zellmembran Kationenkanäle formen und die an ein G-Protein gekoppelten P2Y-Rezeptoren (Burnstock G et al., 1985).
Es existieren sieben bekannte P2X-Rezeptoruntereinheiten, die als $P2X_1$ bis $P2X_7$ bezeichnet werden (Ralevik V et al., 1998).

Die P2Y-Rezeptoren bilden 10 funktionell charakterisierte Subtypen, von denen 8 im menschlichen Genom vorliegen. Alle P2Y-Rezeptoren sind, wie im vorherigen Abschnitt beschrieben, über die verschiedenen heterotrimeren G-Proteine an Second-Messenger-Kaskaden gekoppelt. Sie werden von den unterschiedlichsten Zelltypen exprimiert: Epithel- und Endothelzellen, Thrombozyten, glatte und quergestreifte Muskelzellen, Neurone, Fibroblasten, Monozyten, Makrophagen und Gliazellen (Di Vergilio F, 1995).

Immunzellen besitzen vor allem den $P2Y_2$-Subtyp auf ihrer Zelloberfläche (Abbracchio et al., 1994), dem eine zentrale Rolle bei der Leukozyten-Adhäsion zugeschrieben wird. $P2Y_2$ spielen zudem eine Schlüsselrolle bei der Migration und Orientierung von Zellen unter chemotaktischer Stimulation (Weisman et al., 1996). Humane neutrophile Granulozyten

setzen beispielsweise ATP frei, durch welches der $P2Y_2$ Rezeptor eine Zellausrichtung vermittel kann.

Die ADP-Thrombozytenrezeptoren $P2X_1$ und $P2Y_1$

Der $P2X_1$-Rezeptor ist, wie zuvor beschrieben, ein ligandengesteuerter Kationenkanal, der bei Aktivierung einen sofortigen Kalziumeinstrom von extrazellulär nach intrazellulär bewirkt. Seine genomische Sequenz ist auf dem humanen Chromosom 17 lokalisiert (Valera et al., 1995). Der 399 Aminosäuren umfassende Rezeptor setzt sich aus zwei Transmembrandomänen und einem großen Extrazellulärloop zusammen (Ralevic et al, 1998). Seine Expression ist sehr weit verteilt und wird z.B. in Muskelzellen, Neuronen und Gliazellen exprimiert. Zu den Liganden zählen ATP und ATP-Derivate, wie z.B. αβ-methylen-ATP, aber auch ADP. Seine Aktivierbarkeit durch ADP brachte diesen Rezeptor mit der Thrombozytenaggregation in Verbindung (Sun et al., 1998).
Beim $P2Y_1$-Rezeptor handelt es sich um einen GPCR. Er besteht aus 373 Aminosäuren und ordnet sich in den klassischen sieben TMs an. Er koppelt intrazellulär über ein Gq-Protein (Schachter et al., 1997). Lokalisiert ist seine genomische Sequenz beim Menschen auf Chromosom 3 (Ayyanathan et al., 1996). Der $P2Y_1$-Rezeptor wird weitgehend ubiquitär im Herzmuskel, glatter Muskulatur, Blutgefäßen, Thrombozyten, Hoden, Prostata bzw. Ovarien exprimiert (Ralevic et al., 1998). Zu seinen Liganden zählen ADP und ADP-Derivate, wie d-ADP und 1-Me-ADP. Die Wirkung von ATP wird sowohl als Agonist (Palmer et al., 1998) als auch als Antagonist (Leon et al. 1997) beschrieben. Bei einer Aktivierung bewirkt der $P2Y_1$ ebenfalls einen Kalziumanstieg in der Zelle, ausgelöst durch eine Freisetzung aus intrazellulären Speichern.

Der ADP-Rezeptor $P2Y_{12}$

Der $P2Y_{12}$ setzt sich aus 342 Aminosäureresten zusammen und seine genomische Sequenz ist, wie auch die des Thrombozytenrezeptor $P2Y_1$, auf Chromosom 3 lokalisiert. Der ADP Rezeptor $P2Y_{12}$ ist ein Gi-Proteingekoppelter Rezeptor und seine Aktivität triggert neben anderen Faktoren die Thrombozytenaggregation (Hollopeter et al., 2001). Inaktivierende Mutationen im $P2Y_{12}$-Gen führen nachweislich beim Hund, der Maus und dem Menschen zu funktionellen Blutgerinnungsstörungen (Boudreaux and Martin, 2011; Cattaneo, 2005; Cattaneo et al., 2003; Daly et al., 2009; Fontana et al., 2009; Remijn et al., 2007; Shiraga et al., 2005). Der erste Patient mit einem kongenitalen $P2Y_{12}$-Rezeptordefekt wurde 1992 beschrieben (Cattaneo et al., 1992). Aufgefallen war er durch lebenslang starke Blutungsneigung und postoperative Komplikationen. Seine Blutungszeit betrug 15-20 min und labordiagnostisch konnten selbst hohe ADP-Konzentrationen über 10 µm nicht zu einer irreversiblen Thrombozytenaggregation führen. Der exakte Gendefekt dieser autosomal rezessiv vererbten Erkrankung ist nur bei wenigen Patienten bekannt. Bei einem Geschwisterpaar konnte beispielsweise eine homozygote Mutation 378delC nachgewiesen werden (Cattaneo, 2011). Der $P2Y_{12}$ wird nicht nur auf Thrombozyten, sondern auch in Subregionen des Gehirns exprimiert (Hollopeter et al., 2001). Gliazellen scheinen hierbei eine wichtige $P2Y_{12}$-tragende Zellpopulation darzustellen. Sein fehlen führt zu einer reduzierten Mikrogliaaktivierung (Haynes et al., 2006).

Der $P2Y_{12}$ kann durch ADP (Abb. 1) und hochpotent durch das ADP-Derivat MeS-ADP aktiviert werden. Die Rolle von ATP wird, wie auch beim $P2Y_1$, kontrovers diskutiert und reicht vom Agonismus (Barnard and Simon, 2001; Simon et al., 2002) bis zum Antagonismus (Bodor et al., 2004; Springthorpe et al., 2007).

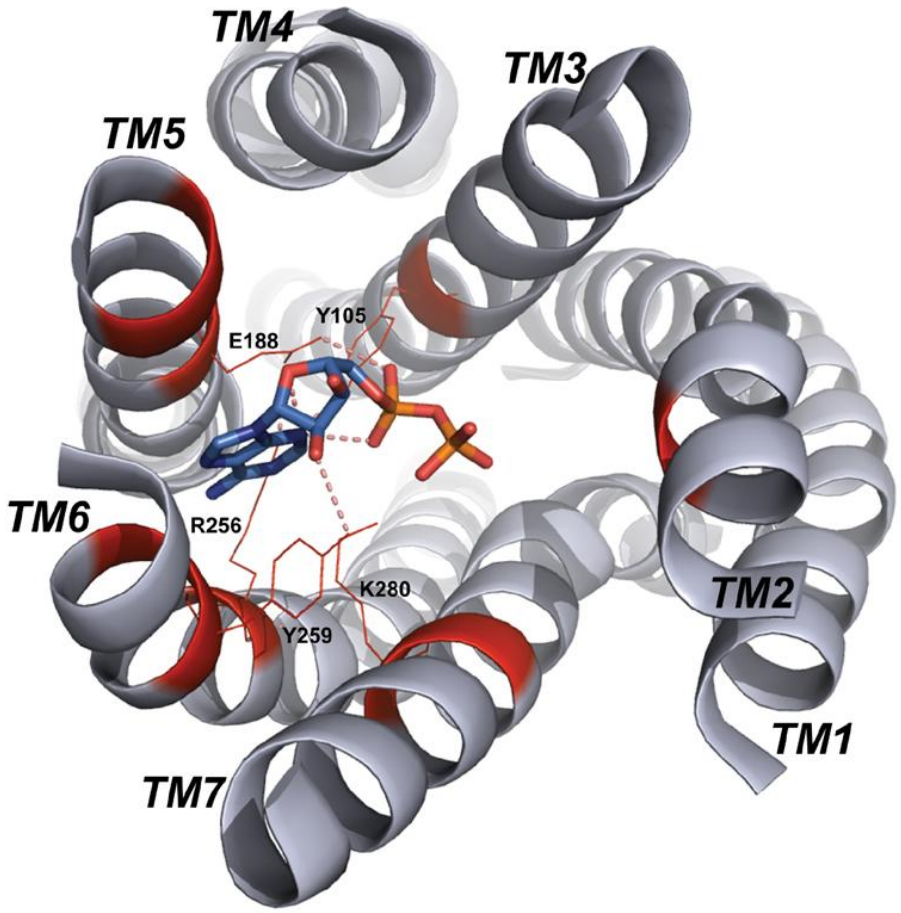

Abb. 1: Strukturmodell des $P2Y_{12}$. Gezeigt ist ADP als Liganden in einem $P2Y_{12}$ Dockingmodell. Rot dargestellt sind die für die Interaktion wesentlichen Aminosäuren. Das Modell ist im Rahmen der nachstehenden Publikation entstanden und dort im Detail beschrieben.

Der ADP-Rezeptor $P2Y_{12}$ steht namensgebend für eine Gruppe strukturell ähnlicher Rezeptoren. Die $P2Y_{12}$-like Gruppe beinhaltet die ADP-Rezeptoren $P2Y_{12}$ (Hollopeter et al., 2001) und $P2Y_{13}$ (Zhang et al., 2002), sowie den UDP-Glukose-Rezeptor $P2Y_{14}$ (Chambers et al., 2000) und die orphanen Rezeptoren GPR82, GPR87, GPR171 (Lee et al., 2001) und GPR34 (Marchese et al., 1999; Schöneberg et al., 1999) (Abb. 2).

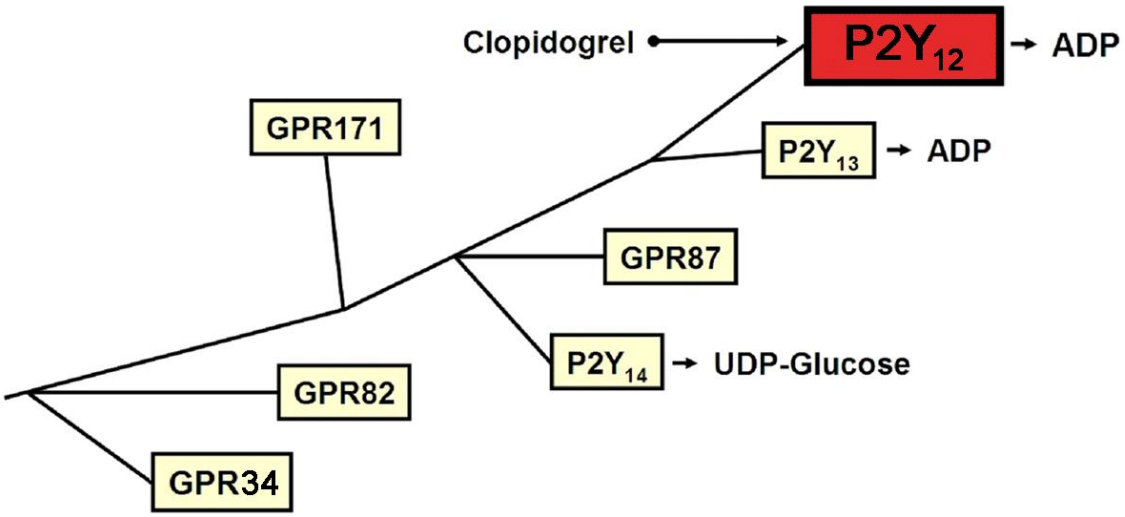

Abb. 2: Phylogenetische Beziehung von Mitgliedern der P2Y12-ähnlichen GPCR. Auf der Grundlage ihrer Aminosäuresequenz wurde ein phylogenetischer Baum erzeugt (Schöneberg et al., 2007). Weiterhin gezeigt sind die bekannten natürlichen Agonisten sowie Clopidogrel als Antagonist am $P2Y_{12}$.

Die Pharmakologie des $P2Y_{12}$

Auf Grund seiner hohen physiologischen Relevanz bei der Thrombozytenaggregration ist der $P2Y_{12}$ ein zentraler Angriffspunkt für antithrombotische Medikamente, wie z. B. Ticlopidin und Clopidogrel. Diese Thienopyridinderivate zeigen eine signifikante Wirkung zur Prophylaxe zerebraler und kardialer ischämischer Ereignisse. Die antagonistischen Eigenschaften dieser Medikamentengruppe stehen im Zusammenhang mit irreversiblen Interaktionen zwischen extrazellulären Cysteinbrücken des $P2Y_{12}$ und dem Pharmakon (Ding et al., 2003). Hierdurch werden aktivierte und inaktive Thrombozyten gehemmt. Die $P2Y_{12}$-Blockade bewirkt über einen intrazellulären Signalweg eine indirekte Hemmung des Glycoprotein IIb/IIIa-Komplexes, welcher für die Bindung von Fibrinogen verantwortlich ist. Ticlopidin und Clopidogrel unterscheiden sich in ihrer Molekülstruktur lediglich um eine Seitengruppe. Clopidogrel wird jedoch bevorzugt eingesetzt, da es im Gegensatz zu seinem Vorgänger keine Leukozytopenien auslöst. Einen besonderen Stellenwert nimmt Clopidogrel bei der Postprophylaxe von koronaren Stent-

Implantationen ein. Sowohl bei Clopidogrel als auch bei Ticlopidin handelt es sich um Prodrugs, welche das Cytochrome P450 2C19 Enzym (CYP 2C19) für die Umwandlung in einen aktiven Metaboliten benötigen. Pharmaka werden als Prodrugs verabreicht, wenn sie in ihrer aktiven Form nicht oral resorbiert werden können, sie dadurch einem hohen First-Pass-Effekt (Metabolisierung des Pharmakons während der ersten Passage durch die Leber) entgehen oder die unerwünschten Wirkungen durch gezielte Metabolisierung am Wirkort gering halten. Problematisch sind Wechselwirkungen mit anderen chemischen Substanzen und Medikamenten, welche ebenfalls durch das CYP2C19 metabolisiert werden und so den Wirkspiegel des Thrombozytenaggregationshemmers verändern. Protonenpumpeninhibitoren, wie Omeprazol, hemmen CYP2C19 und verringern den Wirkspiegel von Clopidogrel. Im Gegensatz dazu induziert das Antibiotikum Rifampicin die Expression von CYP2C19. Aber auch Clopidogrel selbst kann mit anderen Medikamenten gefährlich interagieren. Es verdrängt durch seine hohe Affinität am CYP2C19 das Antiepileptikum Phenytoin und verhindert dessen Abbau. Obwohl Clopidogrel sehr spezifisch und effektiv wirkt, unterliegt es einer hohen Variabilität in der Thrombozytenaggregation, welche genetischen Polymorphismen und Medikamenteninteraktionen geschuldet ist (Munoz-Esparza et al., 2011; Nawarskas and Clark, 2011).

Diese Tatsache führte zur Suche nach alternativen $P2Y_{12}$-Blockern wie Cangrelor, Prasugrel und Ticagrelor. Bei letzteren handelt es sich um reversibel bindende ATP-Derivate (Storey, 2011) (Abb. 3).

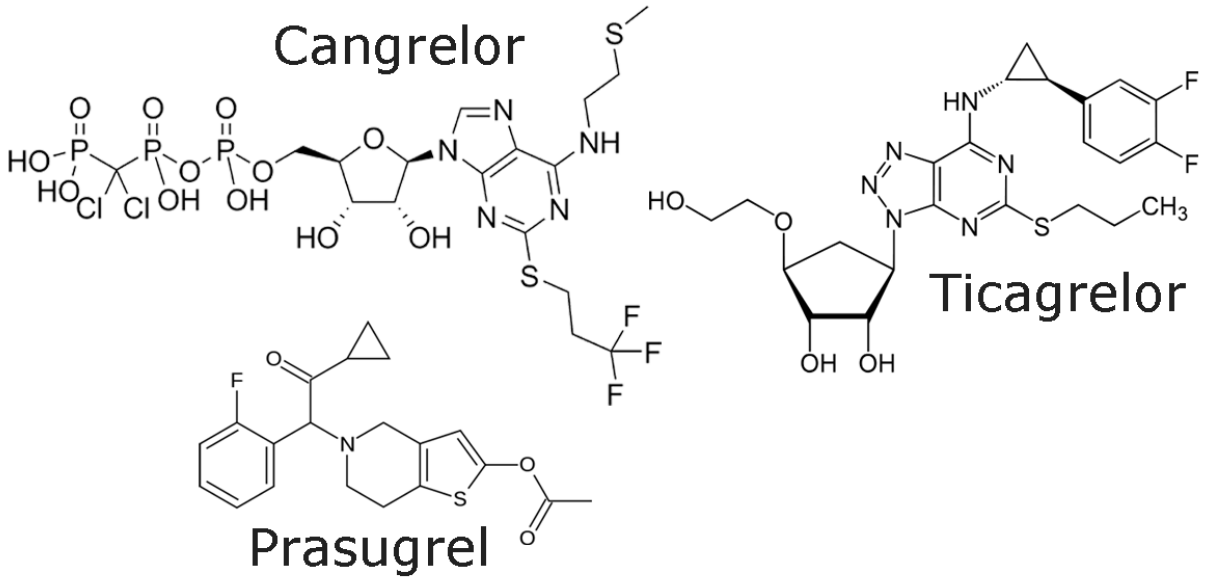

Abb. 3: Strukturformeln von $P2Y_{12}$-Blockern. Cangrelor besitzt das Purinnukleotid ATP als Kernstruktur, bei Ticagrelor ist die 5'-Position der Ribose nicht mehr mit Phosphatgruppen verestert. Das Purin Prasugrel ist strukturchemisch am weitesten von ATP entfernt.

Vorteile und Suche eines inversen Agonisten

Wie alle GPCRs liegt auch der $P2Y_{12}$ in einem Rezeptorgleichgewicht zwischen aktiven und inaktiven Rezeptorkonformationen vor. Liganden und Mutationen in GPCRs können dieses Gleichgewicht beeinflussen. Der $P2Y_{12}$ scheint bereits ohne Agonist in messbarer Menge in aktiven Konformationszuständen vorzuliegen (Chee et al., 2008; Schulz and Schoneberg, 2003). Aus diesem Grund wäre ein inverser Agonist von hoher pharmakologischer Relevanz. Im Gegensatz zu Antagonisten verschieben inverse Agonisten das Gleichgewicht in Richtung inaktive Konformationen. Bisher wurden jedoch erst wenige inverse Agonisten am $P2Y_{12}$ beschrieben (Ding et al., 2006). Im Gegensatz zu klassischen Antagonisten würde ein inverser Agonist nicht nur die Aktivierung des $P2Y_{12}$ verhindern, sondern die Grundaktivität der $P2Y_{12}$-mediierten Aggregation und damit die Thrombenbildung reduzieren helfen.

Die Wahl des geeigneten Expressionssystems

Eine funktionelle Charakterisierung von P2Y-Rezeptoren und ihrer Mutanten in Säugerzellexpressionssystemen ist durchaus problematisch. Gründe hierfür sind die Freisetzungen endogener Nukleotidasen und Nukleotide.

Hefe

Eine Lösung dieser Probleme konnte durch den Einsatz eines heterologen Hefezellsystem erreicht werden. Der humane $P2Y_{12}$ kann in einem Hefezellsystem exprimiert und funktionell getestet werden (Pausch et al., 2004; Schulz and Schoneberg, 2003). Hierbei wird die Rezeptoraktivität des $P2Y_{12}$ oder einer seiner Mutanten über ein chimeres G-Protein direkt an das Zellwachstum gekoppelt. Nur durch die Aktivität des Rezeptors sind die Zellen in der Lage Histidin zu bilden und sich so in einem histidinfreien Medium zu vermehren (Abb. 4). Die Zellvermehrung kann dann mittels optischer Dichte bestimmt werden.

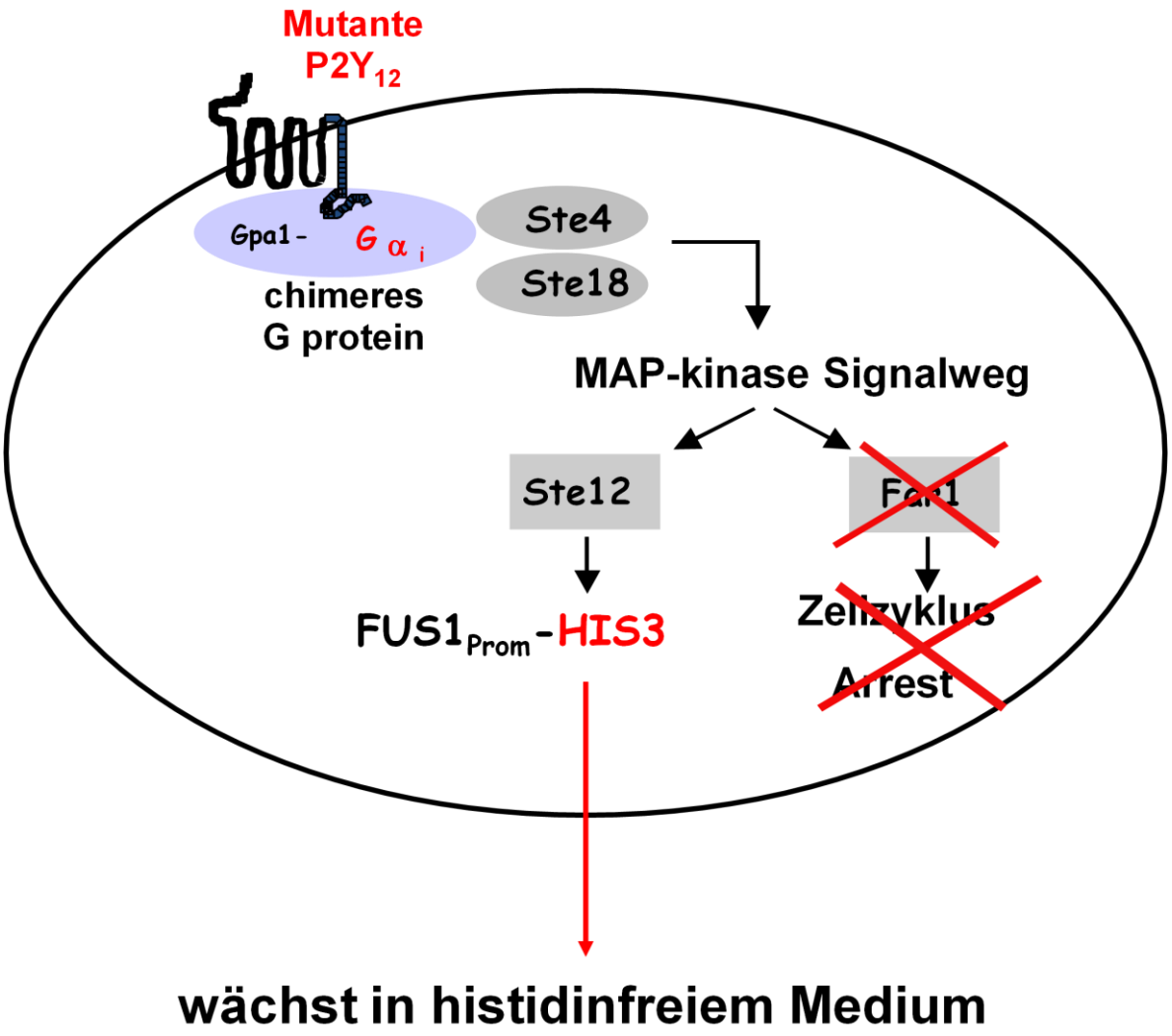

Abb. 4: Signaltransduktion des verwendeten heterologen Hefeexpressionssystems. Gezeigt ist eine $P2Y_{12}$-rezeptorexprimierende Hefezelle. Mit Hilfe eines chimeren G-Proteins wird die Rezeptoraktivität auf einen MAP-kinase Signalweg umgelenkt. Durch den Transkriptionsfaktor Ste12 wird das Reportergen HIS3 Downstream der FUS1-Promotorregion aktiviert. Über die dargestellte intrazelluläre Signalkaskade ist die Hefezelle in der Lage den Zellzyklus Arrest zu umgehen, die Aminosäure Histidin zu produzieren und so im histidinfreien Medium zu leben (Pausch et al., 2004).

Bisher wurden nur sehr wenige aktivierende Mutationen an P2Y Rezeptoren beschrieben (Ding et al., 2006). In einer umfangreichen Mutagenese-Studie wurden aus über 1000 Punktmutationen lediglich 28 konstitutiv aktive $P2Y_{12}$ Mutanten identifiziert (Cöster et al., 2012).

Epic

Eine weitere Möglichkeit des Messverfahrens stellt das dynamische Massenredistributionsverfahren Epic dar. Hierbei handelt es sich um einen Biosensor, welcher agonistvermittelte Zellkinetik optisch detektiert (Fang et al., 2007). Da dies die Möglichkeit offeriert bereits 5 Minuten nach Stimulation zu messen, erlaubt dieser Versuchsaufbau die Rezeptoraktivität abzulesen, bevor die Nukleotide durch chemische oder enzymatische Hydrolyse zerfallen oder umgewandelt werden.

Fragestellung

Der ADP-Rezeptor $P2Y_{12}$ gehört zur Superfamilie der GPCRs und ist an der Vermittlung der Thrombozytenaggregation beteiligt. Da dieser Rezeptor eine erhöhte Basalaktivität besitzt, könnte sich ein therapeutischer Vorteil inverser Agonisten gegenüber Antagonisten ergeben. Bis dato sind nur wenige inverse Agonisten beschrieben. Deshalb galten als wesentliche Ziele der Arbeit mittels einer Purin-Substanzbibliothek Liganden-Struktur-Wirkungsbeziehungen zu ermitteln und darüber neue inverse Agonisten zu identifizieren. Weiterhin sollte mittels computerassistiertem Ligandendocking an einem $P2Y_{12}$-Modell die putative Ligandenbindungstelle identifiziert und über diese Rückschlüsse auf die Potenz von Liganden gezogen werden. Eine systematische, funktionelle Charakterisierung von Liganden sollte Determinanten identifizieren, die eine agonistische und eine invers agonistische Wirkung am $P2Y_{12}$ vermitteln. In Summe möchte diese Arbeit wesentliche Beiträge zum Verständnis von Struktur-Wirkungsbeziehungen am $P2Y_{12}$ leisten.

Publikation

Supplemental Material can be found at:
http://molpharm.aspetjournals.org/content/suppl/2012/10/23/mol.112.082198.DC1.html

1521-0111/83/1/256–266$25.00
MOLECULAR PHARMACOLOGY
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http://dx.doi.org/10.1124/mol.112.082198
Mol Pharmacol 83:256–266, January 2013

Identification of Determinants Required for Agonistic and Inverse Agonistic Ligand Properties at the ADP Receptor $P2Y_{12}$[S]

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Received September 3, 2012; accepted October 23, 2012

ABSTRACT

The ADP receptor $P2Y_{12}$ belongs to the superfamily of G protein–coupled receptors (GPCRs), and its activation triggers platelet aggregation. Therefore, potent antagonists, such as clopidogrel, are of high clinical relevance in prophylaxis and treatment of thromboembolic events. $P2Y_{12}$ displays an elevated basal activity in vitro, and as such, inverse agonists may be therapeutically beneficial compared with antagonists. Only a few inverse agonists of $P2Y_{12}$ have been described. To expand this limited chemical space and improve understanding of structural determinants of inverse agonist-receptor interaction, this study screened a purine compound library for lead structures using wild-type (WT) human $P2Y_{12}$ and 28 constitutively active mutants. Results showed that ATP and ATP derivatives are agonists at $P2Y_{12}$. The potency at $P2Y_{12}$ was 2-(methylthio)-ADP > 2-(methylthio)-ATP > ADP > ATP. Determinants required for agonistic ligand activity were identified. Molecular docking studies revealed a binding pocket for the ATP derivatives that is bordered by transmembrane helices 3, 5, 6, and 7 in human $P2Y_{12}$, with Y^{105}, E^{188}, R^{256}, Y^{259}, and K^{280} playing a particularly important role in ligand interaction. *N*-Methylanthraniloyl modification at the 3′-OH of the 2′-deoxyribose leads to ligands (mant-deoxy-ATP [dATP], mant-deoxy-ADP) with inverse agonist activity. Inverse agonist activity of mant-dATP was found at the WT human $P2Y_{12}$ and half of the constitutive active $P2Y_{12}$ mutants. This study showed that, in addition to ADP and ATP, other ATP derivatives are not only ligands of $P2Y_{12}$ but also agonists. Modification of the ribose within ATP can result in inverse activity of ATP-derived ligands.

Introduction

The ADP receptor $P2Y_{12}$ is a G_i protein–coupled receptor (GPCR) and a key player in platelet aggregation (Hollopeter et al., 2001). Inactivating mutations in $P2Y_{12}$ are responsible for bleeding disorders in humans and dogs (Hollopeter et al., 2001; Cattaneo et al., 2003, 2005; Shiraga et al., 2005; Remijn et al., 2007; Daly et al., 2009; Fontana et al., 2009; Boudreaux and Martin, 2011). With significant relevance in pathophysiology, $P2Y_{12}$ is also the major target of the antithrombotic drugs ticlopidine and clopidogrel. The thienopyridine clopidogrel is a prodrug that requires the cytochrome P450 2C19 enzyme for its conversion to an active thiol metabolite. Several mechanisms of antagonistic action have been proposed for the active metabolite of clopidogrel, including interaction with extracellular cysteine residues of $P2Y_{12}$ (Ding et al., 2003) and receptor dimer disruption (Savi et al., 2006). Although very specific and effective, clopidogrel produces a variable platelet inhibition based on genetic polymorphisms and drug interactions (Munoz-Esparza et al., 2011; Nawarskas and Clark, 2011). This has triggered the search for alternative $P2Y_{12}$ blockers, such as prasugrel, cangrelor, and ticagrelor. The latter two compounds are ATP analogs and bind reversibly at $P2Y_{12}$ (Storey, 2011).

$P2Y_{12}$ displays a high constitutive activity when expressed in vitro (Schulz and Schöneberg, 2003; Chee et al., 2008). Therefore, inverse agonists may be therapeutically beneficial compared with antagonists. Because only a few inverse agonists of $P2Y_{12}$ have been described (Ding et al., 2006), we therefore screened for compounds that reduce the basal activity of constitutively active $P2Y_{12}$ mutants.

This work was supported by the Deutsche Forschungsgemeinschaft [FOR 748, Scho 624/7-1; Sfb 610] and a student fellowship of the Medical Faculty, University of Leipzig, to D.W. Work in the Meiler laboratory is supported by the National Institutes of Health [Grants R01 GM080403, R01 MH090192, and R01 GM099842] and the National Science Foundation (Career 0742762). E.N.D. is supported through the Paul Calabresi Medical Student Research Fellowship from the PhRMA Foundation.
dx.doi.org/10.1124/mol.112.082198.
[S] This article has supplemental material available at mol.aspetjournals.org.

ABBREVIATIONS: $AppNH_2$, adenosine-5′-(amido)diphosphate; AR-C78511, (E)-N-[1-[7-hexylamino)-5-(propylthio)-3H-1,2,3-triazolo-[4,5-d]-pyrimidin-3-yl]-1,5,6-trideoxy-b-D-ribo-hept-5-enofuranuronoyl]-L-aspartic acid; 2I-ATPgammaS, 2-Iodo-adenosine-5′-(-thio)-triphosphate; CHO, Chinese hamster ovary; CXCR4, C-X-C chemokine receptor type 4; dADP, deoxy-ADP; dATP, deoxy-ATP; DMEM, Dulbecco's modified Eagle's medium; DMR, dynamic mass redistribution; GPCR, G protein–coupled receptor; HEK, human embryonic kidney; IP_1, inositol-1-phosphate; LiAc, lithium acetate; MeS-ADP, 2-(methylthio)-ADP; MeS-ATP, 2-(methylthio)-ATP; OD, optical density; PEG, polyethylene glycol; PEP, phosphoenol pyruvate; TNP-ADP, 2′-(OR-3′)-*O*-(trinitrophenyl)-ADP; U^-/H^-, uracil and histidine; WT, wild type.

Functional characterization of P2Y receptors and their mutants in mammalian expression systems is problematic because of the abundance of endogenous nucleotide receptors, nucleosidases, and nucleotide release. In previous experiments, we and others demonstrated that the human P2Y$_{12}$ is functionally expressed in the yeast system (Schulz and Schoneberg, 2003; Pausch et al., 2004), which lacks such problems. Numerous constitutively activating mutations have been described for GPCRs in natural or recombinant systems, but only a few have been reported for P2Y receptors (Ding et al., 2006). From more than 1000 single point mutations, we identified 28 constitutively active P2Y$_{12}$ mutants. Screening a purine compound library, we discovered several new agonists and inverse agonists for the wild-type (WT) P2Y$_{12}$ and constitutively active mutants, respectively.

Materials and Methods

If not stated otherwise, all standard substances were purchased from Sigma-Aldrich (Munich, Germany), Merck (Darmstadt, Germany), and Carl Roth (Karlsruhe, Germany). Cell culture material was obtained from Sarstedt. Salmon sperm DNA, 2-(methylthio)ADP (MeS-ADP) trisodium salt hydrate, lithium acetate (LiAc) dihydrate, lithium chloride (LiCl), polyethylene glycol (PEG) 3350 (catalog no. P-3640), and apyrase from potato (grade III) were obtained from Sigma-Aldrich. MeS-ADP (P2Y$_{12}$ agonist) was dissolved in water, and aliquots of stock solutions (10 mM) were stored at −20°C. Yeast medium components were purchased from Sigma-Aldrich and from BD Biosciences (Heidelberg, Germany). Restriction enzymes were purchased from New England Biolabs (Frankfurt a. M., Germany), primers were synthesized by Life Technologies (Darmstadt, Germany), and P2Y$_{12}$ mutant libraries were provided by Sloning BioTechnology (Puchheim, Germany). The adenine nucleotide library was from Jena Bioscience (Jena, Germany). For compound details, see http://www.jenabioscience.com/images/7c63e6fc71/LIB-101.pdf. We additionally included adenine, adenosine, GDP, GTP, GTPγS, IMP, and xanthine (all from Sigma-Aldrich) in our pharmacologic screenings (further referred to as "purine compound library").

Generation of P2Y$_{12}$ Mutants. Mutants were generated by subcloning SlonoMax-SINGLE libraries (synthesized double-stranded DNA fragments containing individual mutants, fragment sizes 100–150 bp) via unique endogenous or silently introduced restriction sites. P2Y$_{12}$ mutants were introduced into the yeast expression plasmid p416GPD (provided by Dr. Mark Pausch, Wyeth Research, Princeton, NJ) and transformed into *Escherichia coli* DH5α (Life Technologies). Plasmids from individual clones were isolated (plasmid preparation kit; Promega, Mannheim, Germany), and mutations were identified by DNA sequencing. Because full coverage was not achieved after sequencing of 96 clones, missing mutants (4 mutants per position on average) were generated by polymerase chain reaction–based site-directed mutagenesis using mutant-specific mutagenesis primers.

Expression and Functional Testing of P2Y$_{12}$ Mutants in Yeast and Mammalian Cells. The *Saccharomyces cerevisiae* yeast strain MPY578t5 (provided by Dr. Mark Pausch) was used for yeast expression and functional testing of the P2Y$_{12}$ mutants. Cells were transformed with plasmid DNA using the LiAc/salmon sperm carrier DNA/PEG method. In brief, an overnight culture grown at 30°C in YPAD (yeast extract, peptone, dextrose medium with adenine) was diluted to an optical density of 0.2 at 600 nm ($OD_{600\ nm}$) in 50 ml YPAD. This culture was incubated at 30°C until the $OD_{600\ nm}$ reached 0.7–0.9. Cells were then harvested (2500*g* for 5 minutes at room temperature) and washed once with 25 ml of water. The pellet was dissolved in 700 μl of LiAc (100 mM) and incubated for 10 minutes at 30°C. A pellet of 50 μl from the yeast cell suspension was then mixed with 90 μl of PEG 3350 (50% w/v), 13.5 μl of LiAc (1 M), 18.75 μl of salmon sperm carrier DNA (2.0 mg/ml), 2.75 μl of sterile water, and plasmid DNA (1 μg) before being incubated for 30 minutes at 30°C and then for 30 minutes at 45°C.

For selection of constitutively active clones, cells were plated on agar plates not containing uracil and histidine (U^-/H^-). After incubation at 30°C for 4 days, clones were prepared for concentration-response curves. Cells transformed with P2Y$_{12}$ mutants were precultured for 2 days at 30°C in U^-/H^- with 10 μM MeS-ADP. To remove MeS-ADP, cells were washed twice with water and grown in U^-/H^- overnight without MeS-ADP. The yeast cell suspension was then diluted to an $OD_{600\ nm}$ of 0.1. From this cell suspension, 100 μl was pipetted into each well of a 96-well plate and to this, 100 μl of a 2× agonist solution or medium was added. Background growth was suppressed by the addition of 20 mM 3-aminotriazole. Mutants were screened for growth and/or constitutive activity at 10 μM MeS-ADP. All positive mutants were further evaluated through MeS-ADP concentration-response (growth) curves.

The purine compound library was screened for agonists and inverse agonists at the WT P2Y$_{12}$ and constitutively active mutants. One hundred microliters of the respective yeast cell suspension ($OD_{600\ nm}$, 0.1) was pipetted into each well of a 96-well plate and, to these samples, 100 μl of a 2× ligand solution or medium was added. OD measurements were performed 24 and 48 hours later. Compounds identified as agonists or inverse agonists were further characterized in concentration-response setups. IC_{50} and EC_{50} values were calculated using Prism 4 software (GraphPad Software, Inc., La Jolla, CA).

To determine the stability of ATP in the 24-hour yeast assay, we performed a phosphoenolpyruvate (PEP)/pyruvate kinase test. Thus, human WT P2Y$_{12}$ expressing yeast cells were grown identically as done in the previous assays. One hundred microliters of the respective yeast cell suspension ($OD_{600\ nm}$, 0.1) was pipetted into each well of a 96-well plate, and 100 μl of a 2× ATP solution or medium was added. Further, 2 mM PEP and 2 μl of a pyruvate kinase solution (final 6 U/ml) were added every 5 hours to the experimental solution (the total volume additional contained 100 mM imidazole, 5 mM $MgCl_2$, pH 7.15, to assure proper pyruvate kinase function). In case ADP is formed because of degrading, PEP is used by the pyruvate kinase to produce ATP. Then, the concentration of PEP in the medium was monitored over 24 hours using a coupled optical enzyme test. Thus, 50 μl of yeast medium harvested after 0, 4, and 24 hours (or 1 mM PEP for control purposes) were incubated with 0.7 ml assay buffer (100 mM imidazole, 5 mM $MgCl_2$, pH 7.15), 1.5 μl of lactate dehydrogenase (10 U/ml), 1 μl of pyruvate kinase (10 U/ml), 8 μl of a 100 mM ADP solution, and 8 μl of 2 mM NADH. NADH concentration was determined photometrically at 340 nm.

For expression in mammalian cells, Chinese hamster ovary (CHO)-K1 cells were grown in Dulbecco's modified Eagle's medium (DMEM supplemented with 10% [v/v] fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified 5% CO_2 incubator. A CHO-K1 cell line stably expressing the chimeric G protein Gα_{qi4} (Kostenis et al., 2005) was established. Transient transfection experiments of CHO-K1 cells with the respective P2Y$_{12}$ constructs and inositol-1-phosphate (IP_1) accumulation assays were performed as described (Schulz and Schoneberg, 2003). In brief, Gα_{qi4}-stable cells were seeded into 12-well plates (about 0.15×10^6 cells per well), transiently transfected, and about 48 hours after this labeled with 2 μCi/ml [*myo*-^{3}H]inositol (PerkinElmer Life and Analytical Sciences, Waltham, MA). After a 16 hour-labeling period, cells were washed once with serum-free DMEM containing 10 mM LiCl and then incubated for 60 minutes at 37°C with serum-free DMEM containing 10 mM LiCl with or without a compound. After this time, the assay medium was removed and the reaction was stopped by adding 0.3 ml of 0.1 N NaOH, followed by a 5 minute-incubation at 37°C. The alkaline solution was then neutralized by adding 0.1 ml of 0.2 M formic acid, and the IP_1 fraction was isolated by anion exchange chromatography as described (Berridge, 1983) and counted on a liquid scintillation counter.

For cAMP assays, transfected cells were labeled with [^{3}H]adenine (2 μCi/ml; PerkinElmer and Life and Analytical Sciences) for 12 hours and washed once in serum-free DMEM containing 1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), followed by incubation in the presence of the indicated compounds and forskolin (10 μM) for 1 hour at 37°C. Reactions were terminated by aspiration of the medium and addition of 1 ml of 5% (w/v) trichloroacetic acid. The cAMP content of cell extracts was determined by anion exchange chromatography as described (Salomon et al., 1974).

To measure label-free receptor activation, a dynamic mass redistribution (DMR) assay (Corning Epic Biosensor Measurements; Corning Life Sciences, Lowell, MA) with stably transfected human embryonic kidney (HEK) cells (HEK-FlpIn, P2Y$_{12}$ in pcDNA5/FRT) was performed as described previously (Schroder et al., 2010; Ritscher et al., 2012). Briefly, cells were seeded into fibronectin-coated Epic 384–well microplates (60,000 cells per well) and exposed to the various compounds. In DMR measurements, polarized light is passed through the bottom of the biosensor microtiter plate, and a shift in wavelength of reflected light indicates intracellular mass redistribution triggered by receptor activation. DMR was recorded as a measure of cellular activity over 60 minutes. Agonist-induced DMR is concentration dependent, and concentration-effect curves were calculated from response peak maxima (approximately 6 minutes after adding the compound) of optical traces.

Generation of a P2Y$_{12}$ Comparative Model and Ligand Docking. A comparative model of P2Y$_{12}$ was constructed using the protein structure prediction software package Rosetta, version 3.2 (Leaver-Fay et al., 2011). The x-ray crystal structure of C-X-C chemokine receptor type 4 (CXCR4) (Protein Data Bank ID: 3ODU) (Gupta et al., 2001) was chosen as a template on the basis of its high similarity to P2Y$_{12}$ (e-value of $3e^{-15}$ with a sequence coverage of 90%) according to a search using National Center for Biotechnology Information BLASTP on sequences from the Protein Data Bank (Supplemental Fig. S1). CXCR4 and P2Y$_{12}$ also share a conserved disulfide bond between the N-terminal C17 and C270 in extracellular loop 3 (Deflorian and Jacobson, 2011). The backbone coordinates of CXCR4 were retained in the comparative model of P2Y$_{12}$, whereas the loop coordinates were built in Rosetta using Monte Carlo Metropolis fragment replacement combined with cyclic coordinate descent loop closure. In brief, φ-ψ angles of backbone segments from homologous sequence fragments from the Protein Data Bank are introduced into the loop regions. After the fragment substitution, small movements in the φ-ψ angles are performed to close breaks in the protein chain. The resulting full sequence models were subjected to eight iterative cycles of side chain repacking and gradient minimization of φ, ψ, and χ angles Rosetta Membrane (Yarov-Yarovoy et al., 2006).

Ligand docking into the comparative model of P2Y$_{12}$ with ADP, ATP, MeS-ADP, MeS-ATP, mant-ADP, mant-ATP, mant-deoxy-ATP [dATP], and mant-deoxy-ADP was performed with Rosetta Ligand (Meiler and Baker, 2006; Davis and Baker, 2009). Each ligand was allowed to sample docking poses in a 5-Å radius centered at the putative binding site for ADP, determined by averaging the coordinates of critical residues for ligand recognition: R^{256}, Y^{259}, and K^{280} (Hoffmann et al., 2008). Once a binding pose had been determined by the docking procedure, 100 conformations of the ligand created by Molecular Operating Environment (Chemical Computing Group, Toronto, ON, Canada) were tested within the site. Side-chain rotamers around the ligand were optimized simultaneously in a Monte Carlo Metropolis–simulated annealing algorithm. The energy function used during the docking procedure contains terms for van der Waals attractive and repulsive forces, statistical energy derived from the probability of observing a side-chain conformation in the Protein Data Bank, hydrogen bonding, electrostatic interactions between pairs of amino acids, and solvation assessing the effects of both side-chain/side-chain interactions and side-chain/ligand interactions. For each ligand, more than 3000 docked complexes were generated and clustered for structural similarity using bcl::Cluster (Alexander et al., 2011). The lowest energy binding poses from the five largest clusters for each ligand were used for further analysis. The change in free energy with and without ligands bound to P2Y$_{12}$ was calculated for each residue in the receptor. Residues with the greatest difference in predicted energy are suggested to be important for ligand interaction (Supplemental Fig. S2).

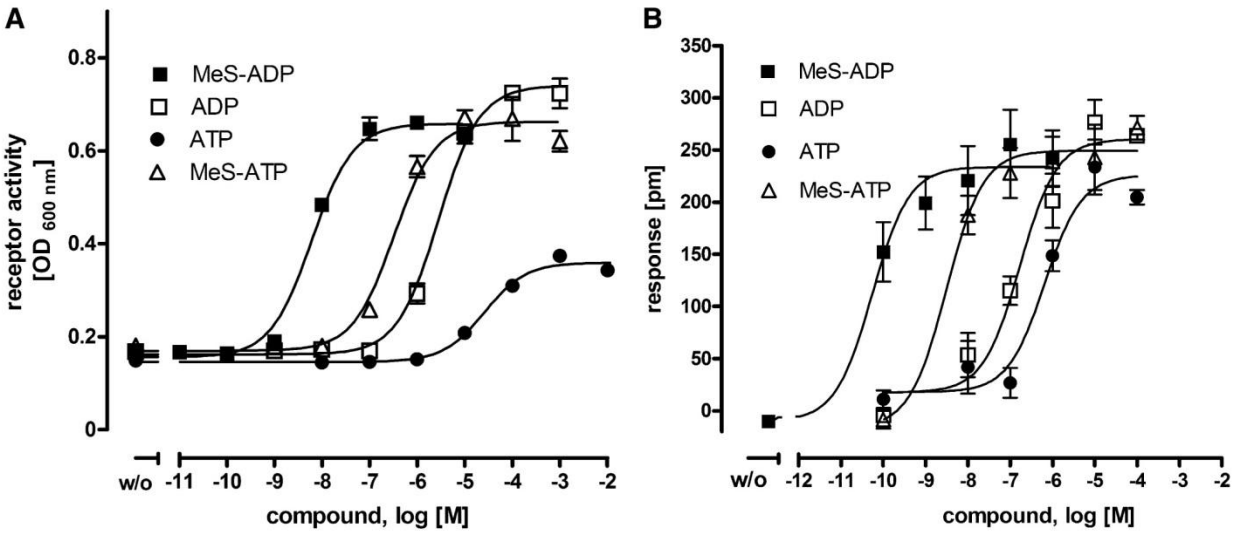


Fig. 1. Nucleotide agonists at the human P2Y$_{12}$. (A) the human P2Y$_{12}$ was transformed into yeast cells and incubated with different concentrations of P2Y$_{12}$ agonists. Receptor activation-dependent growth was measured as OD$_{600\ nm}$ after 24 hours. Data are given as mean ± S.D. of three independent experiments, all performed in triplicate. (B) for label-free measurements of receptor activation, a dynamic mass redistribution assay (Epic Biosensor Measurements) with stably transfected HEK cells was performed essentially as described previously (Schroder et al., 2010). The response is shown 6 minutes after compound application. The response of each compound at nontransfected HEK cells was subtracted from the respective response at P2Y$_{12}$ transfected HEK cells. Data are presented as mean ± S.E.M. of two independent experiments, each carried out in triplicate.

Results

Expression of the Human P2Y$_{12}$ in Yeast and Determination of Agonist Specificity. In previous experiments, we and others have already demonstrated that human P2Y$_{12}$ can be functionally expressed in the yeast system. In this system, P2Y$_{12}$-expressing yeast grows in 96-well cell plates and regular OD measurements are taken. OD values measure cell growth, which is used as a strong indicator for receptor activity.

The WT P2Y$_{12}$ was functionally tested with the compound library for agonists. P2Y$_{12}$ expressed in yeast showed a similar EC$_{50}$ value for MeS-ADP (EC$_{50}$ value, 6 nM; Fig. 1) as when expressed in mammalian cells ranging from low nanomolar to 25–80 nM concentrations (Zhang et al., 2001; Simon et al., 2002; Bodor et al., 2003; Zhong et al., 2004; Ding et al., 2006; Hoffmann et al., 2008). ADP was approximately 500-fold less potent to MeS-ADP, which is consistent with previous findings showing an approximately 30-fold to 1000-fold lower potency in mammalian cells (Zhang et al., 2001; Simon et al., 2002; Bodor et al., 2003). In addition to the highly potent agonist MeS-ADP, we identified additional P2Y$_{12}$ agonists: ATP and MeS-ATP. ATP was a partial agonist at human P2Y$_{12}$ when expressed in yeast (Fig. 1A). EC$_{50}$ values were ranked: MeS-ADP < MeS-ATP < ADP < ATP. We considered the possibility that the registered ATP activity might be due to the fraction of the nucleotides converted to ADP derivatives by nucleotidases or hydrolysis and quantified the possible decay of ATP during the 24-hour assays. Thus, we indirectly quantified ATP degradation in the assay using the PEP/pyruvate kinase system. The pyruvate kinase catalyzes the transfer of a phosphate group from PEP to ADP, yielding one molecule of pyruvate and one molecule of ATP. PEP concentration in the medium is therefore a measure for degraded ATP (see *Materials and Methods*). We found that PEP concentration in the yeast medium remained almost unchanged during 24-hour yeast growth ($\Delta E_{0\ h} = 0.44$; $\Delta E_{4\ h} = 0.44$; $\Delta E_{24\ h} = 0.48$). Only 3.4% of PEP (initial concentration 2 mM) was used by pyruvate kinase for ATP generation. This indicated high stability of ATP (96.6%) in the assay over 24 hours. The functionality of the pyruvate kinase to convert ADP to ATP was verified in control experiments performed in parallel.

The agonistic properties of the adenine nucleotides were verified in the mammalian cell line COS-7 and CHO cells (data not shown), wherein the human P2Y$_{12}$ was coexpressed with the chimeric Gα_{qi4} protein, which redirects receptor activation to the phospholipase C/inositol phosphate pathway (Kostenis et al., 2005). Because ATP produces a cellular response via endogenous nucleotide receptors in most cell lines, we performed additional measurements of P2Y$_{12}$ activation on stably transfected mammalian HEK with a dynamic mass redistribution assay (Epic Biosensor Measurements) (Schroder et al., 2010). Responses of endogenous nucleotide receptors were subtracted from the specific response of P2Y$_{12}$-transfected cells. As shown in Fig. 1B, the concentration-response curves were similar to the data from the yeast expression system except we found that ATP was a full agonist in this mammalian expression system. We also performed Epic measurements in P2Y$_{12}$-stably transfected astrocytoma cells 1321N1, which should not express nucleotide receptors (Filtz et al., 1994). However, ATP-mediated responses in 1321N1 cells were less than those in HEK cells, having no advantage over transfected HEK cells. In sum, the yeast expression system is free of endogenous nucleotide receptors and, therefore, the most straightforward system to use in analyzing P2Y receptors. P2Y$_{12}$ expressed in yeast displays pharmacologic properties very similar to those of mammalian expression systems. Our screening revealed additional compounds with agonistic activity at P2Y$_{12}$: ADPβS, 2′-(OR-3′)-*O*-(trinitrophenyl) (TNP)-ADP, ATPγS, 2I-ATPγS [2-Iodo-adenosine-5′-(-thio)-triphosphate], and adenosine-5′-(amido)diphosphate (AppNH$_2$) (Table 1). We did not follow the pharmacology of these ADP and ATP derivatives further, but they support the fact that derivatives of ATP, as well as of ADP, also have agonistic activities at the human P2Y$_{12}$.

It should also be noted that many nucleotides and nucleosides (e.g., AMP, GTP, cAMP, adenosine) that do not activate P2Y$_{12}$ in mammalian expression systems (Zhang et al., 2001) did not activate P2Y$_{12}$ expressed in yeast.

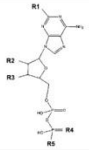
The comprehensive compound library allowed identification of all determinants necessary for agonist function at P2Y$_{12}$ (Supplemental Table 1). Compounds showing significant agonistic activity are given in Table 1. Substitutions that are not compatible with agonistic activity at the human P2Y$_{12}$ (at least in two tested compounds) are listed in Table 2. The results for agonistic activity can be roughly summarized for the three major nucleotide components (base, ribose, phosphate groups):

1. The purine ring is absolutely required. Some modifications (methylthio, iodo) at the 2-position of adenine

TABLE 1
Mant-dATP is an inverse agonist at different constitutively active mutants

Position	Mutation	Inverse Activating Substances (Fold over Basal ≤ 0.8)
F^{246}	V	mant-dATP
F^{254}	I	mant-dATP, mant-N^6-methyl-ATP
	L	mant-dATP, mant-N^6-methyl-ATP
	V	mant-dATP
F^{296}	A	mant-dATP
	C	mant-dATP, mant-N^6-methyl-ATP
	M	mant-dATP
F^{300}	N	mant-dATP
L^{301}	N	mant-dATP
	M	mant-dATP

TABLE 2
Structure of compounds with agonistic properties at the WT human P2Y$_{12}$

Backbone	Substances	Side Chain				
		R1	R2	R3	R4	R5
	ADP	H	OH	OH	O	OH
	ADPβS	H	OH	OH	S	OH
	TNP-ADP	H	$C_6N_3O_8H_4$		O	OH
	AppNH$_2$	H	OH	OH	O	NH$_2$
	ATP	H	OH	OH	O	PO_4H_2
	ATPγS	H	OH	OH	O	PSO_3H_2
	2I-ATPγS	I	OH	OH	O	PSO_3H_2

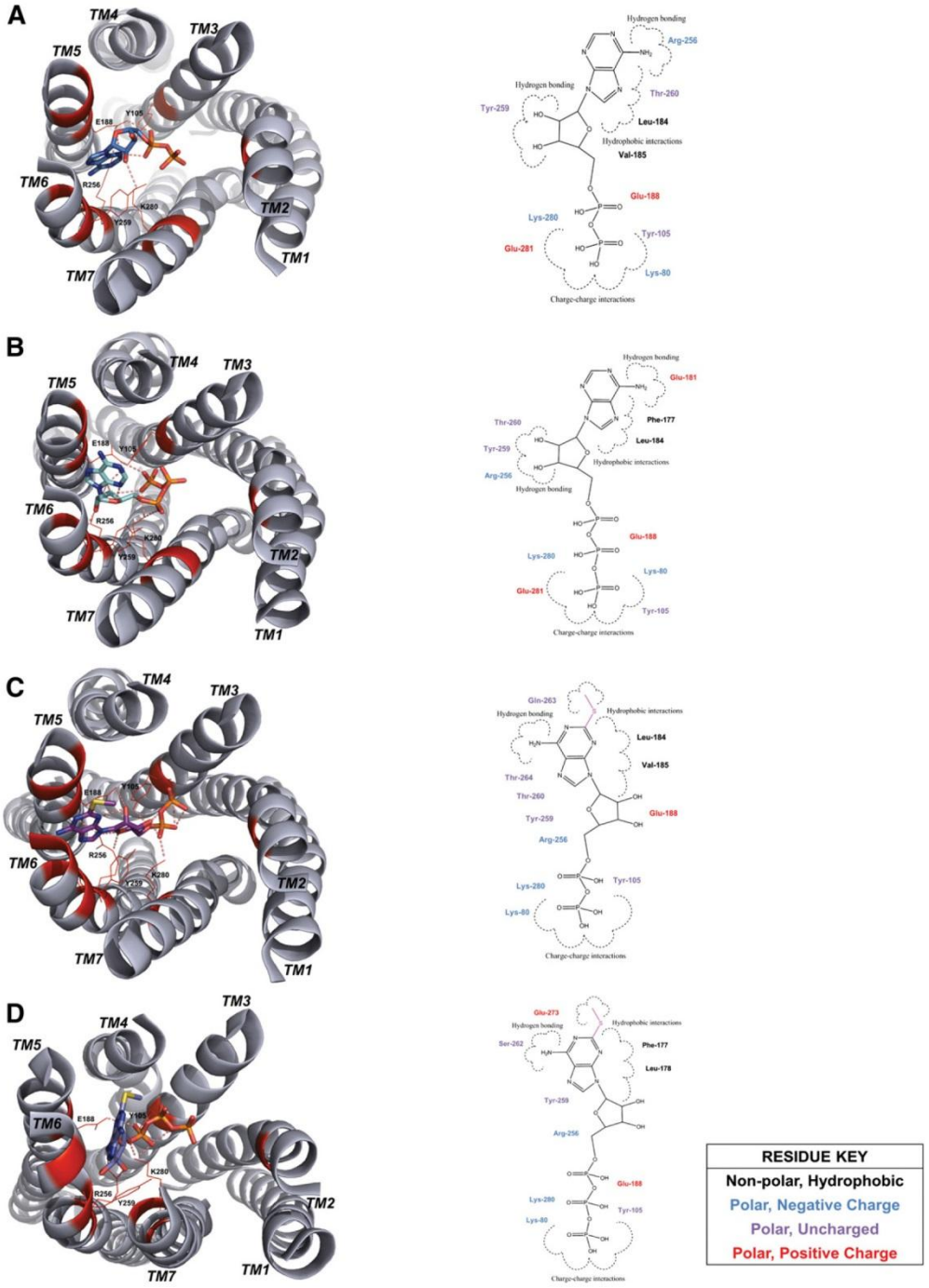


Fig. 2. $P2Y_{12}$ docked in complex with agonists ADP, ATP, MeS-ADP, and MeS-ATP. The docked binding poses in the comparative model of $P2Y_{12}$ for agonists (A) ADP, (B) ATP, (C) MeS-ADP, and (D) MeS-ATP in relation to residues Y^{105}, E^{188}, R^{256}, Y^{259}, and K^{280}. All side chains within the binding site important for ligand interaction according to calculations of free energy change with and without ligands bound to $P2Y_{12}$ are highlighted in red in the model and also shown in relation to the two-dimensional ligand depiction.

are tolerated, but guanine- and inosine-based nucleotides are not agonistic.

2. Deoxidation of the ribose is not tolerated. The trinitrophenyl modification (TNP-ADP) is tolerated.
3. Adenine nucleotides with two or three phosphate residues are agonistic, whereas fewer than 2 phosphate residues or cyclic phosphates are insufficient for agonistic activity. Some substitutions of phosphate

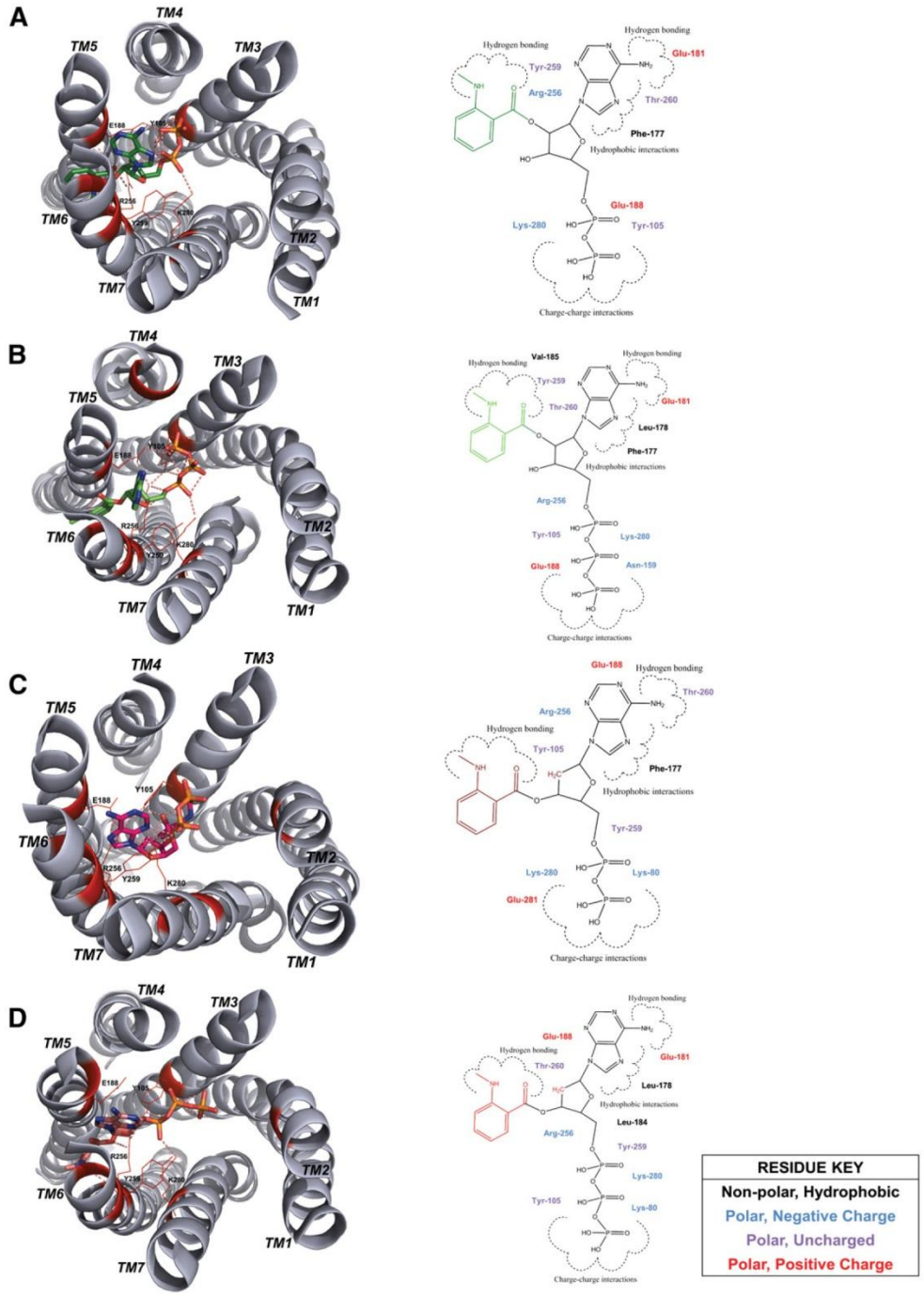


Fig. 3. P2Y$_{12}$ docked in complex with agonists mant-ADP and mant-ATP and inverse agonists, mant-dADP and mant-dATP. The docked binding poses in the comparative model of P2Y$_{12}$ are shown for agonists (A) mant-ADP and (B) mant-ATP and the inverse agonists (C) mant-dADP and (D) mant-dATP in relation to residues Y^{105}, E^{188}, R^{256}, Y^{259}, and K^{280}. All side chains within the binding site important for ligand interaction according to calculations of change in free energy with and without ligands bound to P2Y$_{12}$ are highlighted in red and shown in relation to the two-dimensional ligand depiction.

moieties, as in ADPβS, ATPγS, and AppNH$_2$, are tolerated.

4. Adenine nucleotide multimers (P1-(5′-adenosyl) P3-(5′-adenosyl) triphosphate, P1-(5′-adenosyl) P4-(5′-adenosyl) tetraphosphate, P1-(5′-adenosyl) P5-(5′-adenosyl) pentaphosphate, P1-(5′-adenosyl) P6-(5′-adenosyl) hexaphosphate) displayed no agonistic activity.

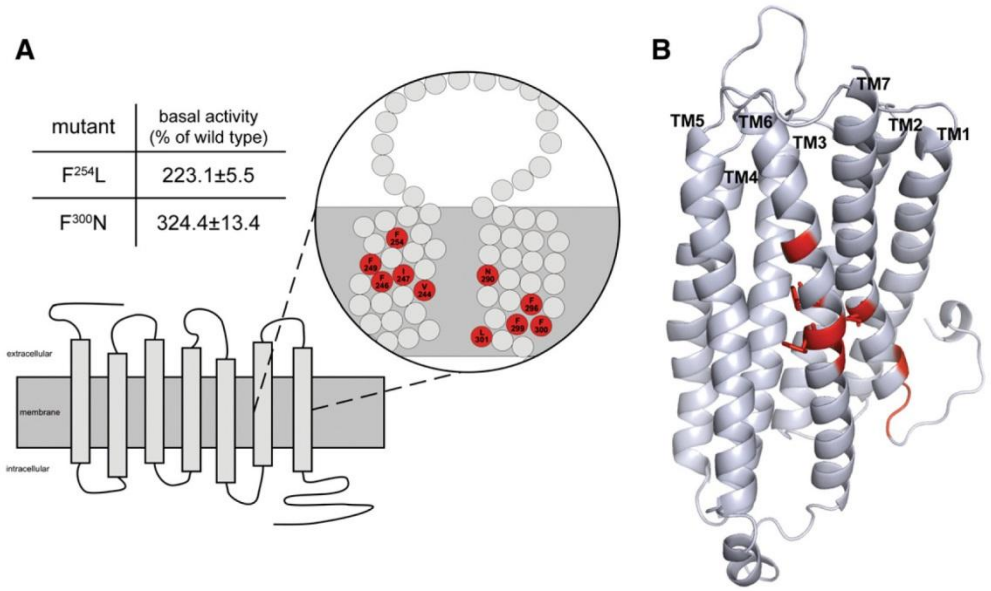


Fig. 4. Position and basal activity of constitutively active $P2Y_{12}$ mutants. (A) the position of constitutively active mutations in transmembranes 1 and 7 (TM6 and TM7) are depicted. Basal activities of the individual mutants expressed in yeast are given in the table. Data are presented as mean ± S.D. of three independent experiments, each carried out in triplicate. The basal activity of the WT $P2Y_{12}$ was $OD_{600\ nm}$: 0.074±0.016. Complete functional data are available and organized in a $P2Y_{12}$ mutant database (http://www.ssfa-7tmr.de/p2y12). (B) the comparative model of $P2Y_{12}$ based on the CXCR4 template is depicted. Residues producing constitutively active mutants on TM6 and TM7 are highlighted in red. Residue side chains facing the pore of the receptor (F^{246}, F^{249}, and N^{290}) are shown in sticks.

Structural Model of Agonist Binding. To estimate whether the different agonists may have similar binding properties, we simulated binding by docking the agonists into the comparative model of $P2Y_{12}$ (Figs. 2 and 3). The model suggested that ADP, ATP, MeS-ADP, MeS-ATP, mant-ADP, mant-ATP, mant-dADP, and mant-dATP bind in the site bordered by transmembrane helices 3, 5, 6 and 7. Ligands were oriented such that the phosphate groups generally pointed toward transmembrane helices 3 and 7, forming hydrogen bonds with Y^{105} and K^{280}. Adenosine rings frequently interacted with the hydrophobic residues on transmembrane helix 5, namely L^{184}, V^{185}, and F^{177} in the second extracellular loop. In agreement with previous docking studies, R^{256} and K^{280} were found to be critical residues in the ADP binding pocket (Deflorian and Jacobson, 2011; Ignatovica et al., 2011). R^{256} frequently interacts with the hydroxyl groups and the oxygen from the furanose. K^{280} is demonstrated to interact with the negatively charged phosphate groups of the ligands. In addition to the R^{256} and K^{280}, Y^{105}, E^{188}, and Y^{259} are consistently found to interact with the ligand. Y^{105} and E^{188} form hydrogen bonds with the phosphate tail, whereas Y^{259} seems to stabilize the adenine.

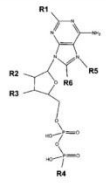
Identification of Constitutively Active Mutants. It is still impossible to predict mutations leading to constitutive activity of a given GPCR. Furthermore, at positions where some mutations activate the GPCR, not all mutations will result in constitutive receptor activation (Lalueza-Fox et al., 2007; Bakker et al., 2008). Therefore, screening of mutant libraries is required. Mutations induced via error-prone derived mutant libraries cannot provide mutational saturation of every codon, and instead, most alleles will contain more than one mutated codon (Li et al., 2007; Thor et al., 2008). Recent advances in gene synthesis technology (see *Materials and Methods*) have made it possible to generate comprehensive mutant libraries.

Here, we mutated every single position to all possible amino acids in a receptor region known from other GPCRs to be sensitive for mutational induced constitutive activity. In sum, 1254 $P2Y_{12}$ mutants were generated covering 66 positions (amino acid positions 236–301) of the receptor and yielding 28 constitutive active mutants at 10 positions (positions are given in Fig. 4). Most mutations were found at positions that faced the lipid, whereas three positions faced the receptor pore (F^{246}, F^{249}, and N^{290}) and three were near the C-terminal receptor tail (F^{299}, F^{300}, and L^{301}). All data are available and organized in a $P2Y_{12}$ mutant database (http://www.ssfa-7tmr.de/p2y12).

Identification of Mant-dATP as Inverse Agonist at Constitutively Active $P2Y_{12}$ Mutants. Constitutively active mutants were expressed and the purine compound library was tested for inverse agonists. *N*-methyl-anthraniloyl- (mant-) dATP reduced basal activity of many constitutively active $P2Y_{12}$ mutants (Table 3). For several mutants, mant-N^6-methyl-ATP was also an inverse agonist (see Table 3). There is no obvious structural overlap or difference between the mutants at which the different inverse agonists act or do not act.

Inverse agonist activity was studied at $F^{254}L$ in more detail. As shown in Fig. 5A, mant-dATP suppressed basal activity in a concentration-dependent manner, with an IC_{50} value in a micromolar range. Of note, the potency of mant-dADP was lower than that of mant-dATP (see Fig. 5A). Both the deoxy- and the mant- modifications are required because mant-ATP and dATP had no effect on basal activity of P2Y12 mutants. It

TABLE 3
ADP modifications not compatible with agonistic activity at the WT human $P2Y_{12}$

Backbone	Position	Side Chain
	R1	OH
	R2	H
	R3	H
		CH_3
		I
		Br
	R4	NH_2
	R5	CH_3
	R6	Br

The table summarizes the modifications at the ADP backbone that are not compatible with agonistic activity at the WT human $P2Y_{12}$ at in least two different adenine nucleotides of the compound library (see *Materials and Methods*).

should be noted that the basal activities of several mutants ($V^{244}E$, $F^{246}C$, $F^{246}G$, $F^{246}P$, $F^{246}S$, $F^{246}T$, $I^{247}F$, $F^{249}Y$, $N^{290}W$, $N^{290}Y$, $F^{296}I$, $F^{296}L$, $F^{296}V$, $F^{299}I$, $F^{299}V$, $L^{301}C$, $L^{301}G$, and $L^{301}T$) were not reduced by mant-dATP or any other compound tested. It is known already that WT $P2Y_{12}$ displays increased basal activity compared with nontransfected mammalian cells (Schulz and Schoneberg, 2003).

To verify that mant-dATP mediates its inverse agonistic activity at the constitutive activity of the WT $P2Y_{12}$ expressed in mammalian cells as well, CHO-K1 cells were co-transfected with chimeric $G\alpha_{qi4}$ and IP_1 accumulation assays were performed. As shown in Fig. 5B, the WT $P2Y_{12}$ displayed a high basal activity and MeS-ADP increased IP_1 levels only 2-fold. Mant-dATP almost completely blocked basal IP_1 formation at the WT $P2Y_{12}$ and $F^{300}N$ (Fig. 5B). Also in cAMP inhibition assays at CHO-K1 cells, mant-dATP displayed strong inverse agonistic activity on the inhibition of basal cAMP formation at the WT $P2Y_{12}$ (Fig. 5C).

Some cell lines release receptor function–relevant amounts of nucleotides into the cell culture medium (Parr et al., 1994; Lazarowski et al., 1997). This may account for high basal activity of $P2Y_{12}$ heterologously expressed in mammalian cell lines. Therefore, we performed similar control experiments with CHO-K1 cells stably transfected with $G\alpha_{qi4}$. As shown in Supplemental Fig. S3, $G\alpha_{qi4}$-CHO-K1 cells transiently transfected with $P2Y_{12}$ presented an increased basal IP_1 level compared with cells transfected with the control plasmid (GFP). Incubation with apyrase did not reduce this elevated IP_1 level. This finding clearly indicates that $P2Y_{12}$ does induce signal transduction by intrinsic active receptor conformation and not by nucleotides released from the cells into the medium. Proper apyrase function was demonstrated by loss of ADP action on $P2Y_{12}$.

Mant-dATP Is Most Likely an Orthosteric Ligand at $P2Y_{12}$. To evaluate whether mant-dATP mediates its inverse agonistic action through an orthosteric or an allosteric binding site, the ADP concentration-response curves at F254L were determined in the presence of different concentrations of mant-dATP. As shown in Fig. 6A, increasing concentrations of mant-dATP shifted the concentration-response curves to higher ADP concentrations. Similar results were obtained for mant-dADP, but with lower potency (Fig. 6B). This competition indicates an orthosteric binding modus for the inverse agonists. Although functional and docking data (see below) support orthosteric binding, we cannot rule out the possibility of an allosteric binding of the inverse agonists given the limited concentration range of mant-dATP investigated herein.

Structural Model of Inverse Agonist Binding. Using our $P2Y_{12}$ model, we investigated whether mant-dATP can dock into the agonist binding pocket of $P2Y_{12}$ and whether specific interactions may explain inverse agonistic activity (Fig. 3). As with the other ATP derivatives, mant-dATP sits between (transmembrane helix TMH) 3, 5, 6 and 7 with Y^{105} and K^{280}, forming hydrogen bonds with the phosphate tail and R^{256} stabilizing the oxygen connecting the furanose to the mant group. Unlike ATP, the extra bulk of the mant group is further stabilized by interactions with I^{257}, H^{253}, and Q^{263}. However, similar interactions are seen with mant-ATP, which does not exhibit inverse agonism. We conclude that the inverse agonistic activity is probably not the result of a different binding pose. It is possibly caused by smaller-scale modulations in the strengths of specific interactions between ligand and protein. Pin-pointing these changes to reveal the mechanism behind the inverse agonistic activity are beyond the accuracy of the present comparative model but will be the focus of future mutational studies.

Discussion

We used a genetically modified yeast strain (Pausch et al., 2004) to heterologously express and functionally test the human ADP receptor $P2Y_{12}$. This expression system offers some advantages over mammalian cells lines, specifically in characterizing nucleotide receptors, because it lacks endogenous nucleotide receptors. ADP and MeS-ADP are full agonists in this expression system, with EC_{50} values of 2.8 μM and 6 nM, respectively (Fig. 1A). Screening a purine compound library, we identified ATP and some derivatives as partial agonists at $P2Y_{12}$ in addition to ADP and its derivatives (Supplemental Table S1). The agonistic activity of ATP was found not only in the heterologous yeast expression system but also in different mammalian cell lines and signaling assays.

That MeS-ATP and ATP bind to the human $P2Y_{12}$ has been shown (Savi et al., 2001), but the ligand properties of ATP at $P2Y_{12}$ are controversial, ranging from antagonism (Bodor et al., 2004; Springthorpe et al., 2007) to agonism (Barnard and Simon, 2001; Simon et al., 2002). These contrary results are probably due to differences in mammalian expression systems and functional assays used. Introduction of a 2′-methylthio group increased ligand potency at $P2Y_{12}$ and made ATP a highly potent full agonist (Fig. 1A), consistent with previous findings (Zhang et al., 2001; Simon et al., 2002). Through ligand docking into a structural comparative model of $P2Y_{12}$, ATP derivatives are found to bind in a similar binding site. Although our structural $P2Y_{12}$ model is not at the resolution to reveal what fine-structural requirements are essential to turn a nucleotide into an agonist at $P2Y_{12}$, specific residues critical to ligand interaction can be predicted from the model. Notably, we find that for six of the seven residues indicated to be significant for ligand interaction that are also in the mutant database (H^{253}, I^{257}, Y^{259}, T^{260}, Q^{263}, T^{264}, and K^{280}), mutation of the residues to any other amino acid results in a loss of WT function (see our $P2Y_{12}$ mutant database: http://www.ssfa-7tmr.de/p2y12). Therefore, there is agreement

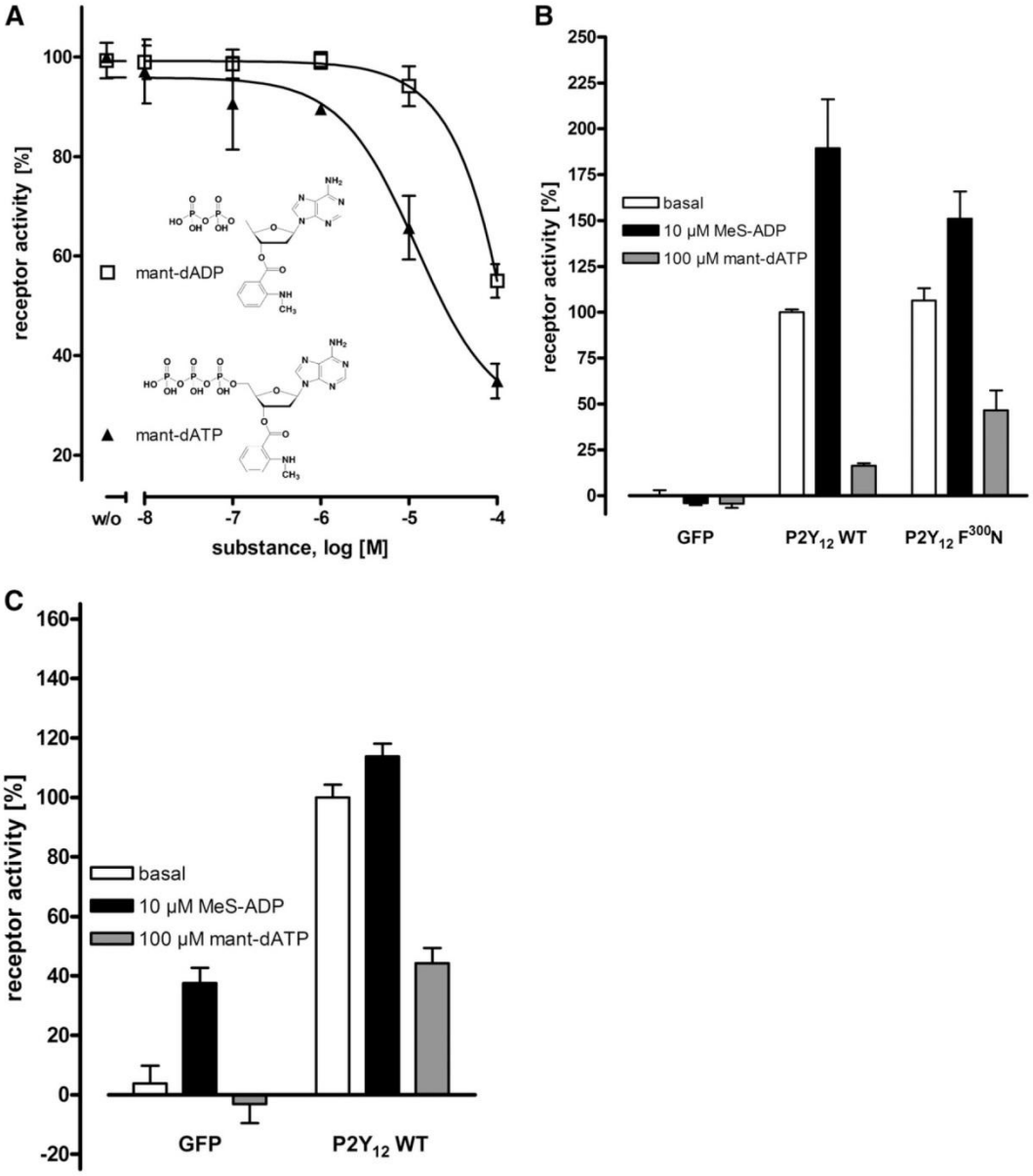


Fig. 5. Mant-dADP and mant-dATP are inverse agonists at constitutively active $P2Y_{12}$. (A) yeast cells expressing $F^{254}L$ were incubated with increasing concentrations of the indicated compounds, and yeast growth was measured after 24 hours' incubation. The ligand-induced decrease of basal activity of $F^{254}L$ is shown relative to the basal activity of the WT $P2Y_{12}$ ($OD_{600\ nm}$, 0.074; set to 0%) and the basal activity of $F^{254}L$ ($OD_{600\ nm}$, 0.165; set to 100%). Data are given as mean ± S.D. of three independent experiments, all performed in triplicate. (B) to evaluate inverse agonist specificity, CHO-K1 cells, stably expressing the chimeric G-protein $G\alpha_{qi4}$, were transfected with plasmids encoding GFP (control), the human ADP receptor or $F^{300}N$. IP_1 formation under basal conditions (white), in the presence of 10 μM MeS-ADP (black bars) and in presence of 100 μM mant-dATP (light gray bars). The basal IP_1 for GFP was 321 cpm per well and set to 0%, the basal IP_1 for WT $P2Y_{12}$ was 970 cpm per well and set to 100%. Data are presented as mean ± S.D. (cpm per well) of three independent experiments, each carried out in duplicate. (C) forskolin-induced cAMP levels in CHO-K1 cells stably expressing human ADP receptor were determined under basal conditions (white bars), in the presence of 10 μM MeS-ADP (black bars), and in presence of 100 μM mant-dATP (light gray bars). The decrease of basal activity of WT $P2Y_{12}$ is shown relative to GFP basal activity (7486 cpm per well; set to 0%) and basal activity of WT $P2Y_{12}$ (4533 cpm per well; set to 100%). Data are given as mean ± S.D. of three independent experiments, all performed in triplicate.

between the residues predicted to be critical for agonist function through docking studies and experimental results. Our model and the docking studies are consistent with the fact that ATP fits into the same binding pocket as well characterized agonists.

These findings raise a relevant question about whether ATP can serve as a $P2Y_{12}$ agonist also in vivo. The ATP-to-ADP ratio in human platelet-dense granules is approximately 2 (Weiss et al., 1979; Cattaneo et al., 2000). If one assumes that ATP and ADP secretion from dense granules occurs with

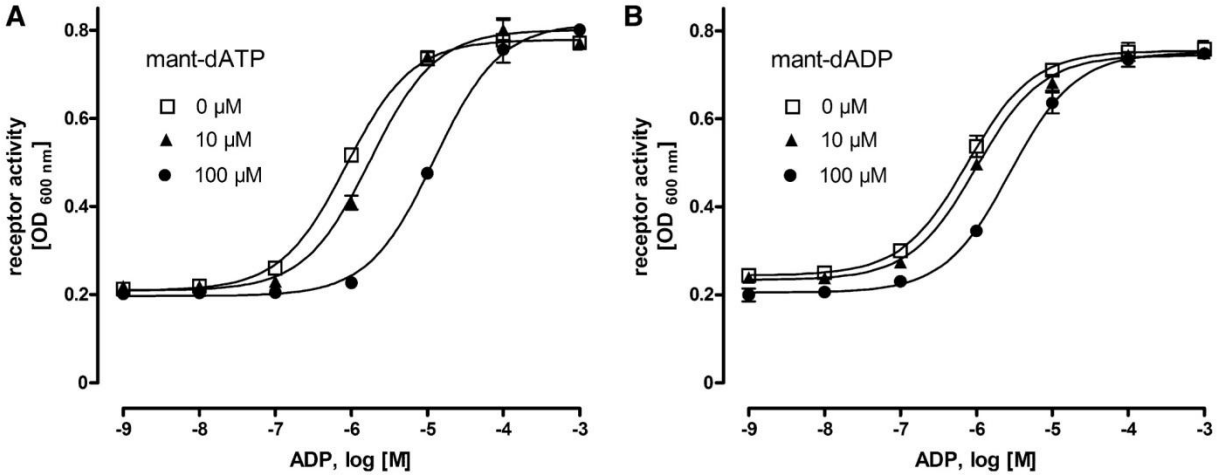


Fig. 6. Mant-dADP and mant-dATP are most likely orthosteric ligands at P2Y$_{12}$ F^{254}L. To evaluate the modus of inverse agonist binding, ADP concentration-response curves at F^{254}L-transformed yeast cells were determined in the presence of 0, 10, and 100 μM mant-dATP (A) and mant-dADP (B). Data are given as mean ± S.D. of three independent experiments, all performed in duplicate. Yeast cells expressing different basal active mutants were incubated with a 10 μM purine compound library to identify inverse agonists. In a screen of more than 80 adenine nucleotides and their derivatives, mant-dATP and mant-N^6-methyl-ATP showed inverse activity on several constitutively active mutants. All the mutants listed showed activation (more than 2-fold above increased basal activity) upon stimulation with ADP, MeS-ADP, ATP, and mant-ADP. dATP and mant-ATP had no significant effects on the mutants.

the same kinetics, previous data suggest that the surface concentration of ADP after thrombin stimulation will transiently reach 7–10 μM (Beigi et al., 1999). This is sufficient for activation of the platelet P2Y$_{12}$ by ADP but also by ATP and is consistent with feed-forward autocrine/paracrine activation of platelet responses.

Many WT GPCRs, such as histamine receptors, thyrotropin receptor, and melanocortin receptors, present high basal activity (Seifert and Wenzel-Seifert, 2002). In contrast to antagonists, inverse agonists suppress both agonist-dependent and -independent activity and are therefore developed in priority. For example, many β-blockers and atropine are inverse agonists at β1-adrenoceptors and muscarinic acetylcholine receptors, respectively (Thor et al., 2009; Baker et al., 2011). Therapeutically used P2Y$_{12}$ ligands are high-affinity antagonists, but inverse activity was described only for the experimental P2Y$_{12}$ blocker AR-C78511 [(*E*)-*N*-[1-[7-hexylamino)-5-(propylthio)-3*H*-1,2,3-triazolo-[4,5-*d*]-pyrimidin-3-yl]-1,5,6-trideoxy-β-D-ribo-hept-5-enofuranuronoyl]-L-aspartic acid] (Vasiljev et al., 2003; Ding et al., 2006). AR-C78511 is a 2-alkylthio-substituted ATP analog but, in contrast to mant-dATP, has no modification at the 2′ or 3′ OH residues of the ribose. Mant-dATP most likely binds at the orthosteric ligand-binding site, and inverse agonistic activity mutually depends on the deoxyribose because mant-ATP lacks inverse agonistic activity. At present we cannot explain or predict inverse activity, even with a receptor model in hand, because the pharmacologic properties of a ligand are the result of a tight interplay of the ligand and the receptor molecule. It is, however, of interest that, as for AR-C78511 (Springthorpe et al., 2007), modification of an ATP backbone resulted again in an inverse agonist (mant-dATP). This also supports our findings that P2Y$_{12}$ naturally recognizes not only ADP but also ATP and that binding of ATP and other ATP derivatives induces conformational changes within P2Y$_{12}$.

In summary, we clearly show that, in addition to ADP and ATP, some ATP derivatives are not only ligands of P2Y$_{12}$ but also agonists. Keeping with an ATP/ADP ratio > 1 in vivo and the small differences in concentration-response curves (Fig. 1B), P2Y$_{12}$ should rather be referred to as an adenine nucleotide receptor without suggesting ADP specificity. Modification of the ribose within ATP can result in inverse activity of ATP-derived ligands.

Authorship Contributions

Participated in research design: Schmidt, Dong, Meiler, Schöneberg.

Conducted experiments: Schmidt, Ritscher, Hermsdorf.

Contributed new reagents or analytic tools: Cöster, Wittkopf.

Performed data analysis: Schmidt, Dong, Ritscher, Hermsdorf, Schöneberg.

Wrote or contributed to the writing of the manuscript: Schmidt, Dong, Meiler, Schöneberg.

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Zusammenfassung der Arbeit

Dissertation zur Erlangung des akademischen Grades Dr. med.

Titel: Identifizierung von agonistischen und invers agonistischen Eigenschaften determinierender Strukturen in Liganden am ADP-Rezeptor $P2Y_{12}$

eingereicht von: Philipp Schmidt

angefertigt an: Universität Leipzig, Medizinische Fakultät
Institut für Biochemie

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eingereicht im: 05/2012

Der zur Superfamilie der GPCRs gehörende ADP-Rezeptor $P2Y_{12}$ ist an der Vermittlung der Thrombozytenaggregation beteiligt und besitzt *in vitro* eine erhöhte Basalaktivität. Aus dieser Tatsache könnte sich ein therapeutischer Vorteil inverser Agonisten gegenüber Antagonisten ergeben. Da bisher lediglich ein inverser Agonist beschrieben war, galt als wesentliches Ziel der Arbeit, mittels einer Purin-Substanzbibliothek Liganden-Struktur-Wirkungsbeziehungen zu ermitteln und darüber neue inverse Agonisten zu identifizieren.
Um den humanen ADP-Rezeptor heterolog zu exprimieren und funktionell zu testen, wurde ein genetisch modifizierter Hefestamm verwendet.

Dieses Expressionssystem offerierte insbesondere für die Charakterisierung von Nukleotidrezeptoren eine Reihe an Vorteilen gegenüber Säugerzelllinien, da es selbst keine endogenen Nukleotidrezeptoren besitzt. In diesem System zeigten die Agonisten ADP und MeS-ADP mit EC_{50} Werten von 2,8 µM und 6 nM die erwartete Potenz und volle agonistische Aktivität. Somit konnte dieses Hochdurchsatz-Expressionssystem äquivalent zu anderen Testsystemen eingesetzt werden. Durch das Testen einer Purinsubstanzbibliothek konnten neben ADP und MeS-ADP auch die ATP-Derivate ATPγS und 2I-ATPγS als Agonisten sowie ATP und MeS-ATP als partielle Agonisten, am $P2Y_{12}$ identifiziert werden. Die agonistische Aktivität für ATP konnte nicht nur im heterologen Hefeexpressionssystem gezeigt werden, sondern auch in verschiedenen Säugerzelllinien und Signalassays. Die in der Literatur kontrovers diskutierten Eigenschaften von ATP am $P2Y_{12}$ sind wahrscheinlich auf sehr unterschiedliche Messsysteme zurückzuführen. Die eigenen, im Rahmen der Arbeit gewonnen, Daten legen nah, dass ATP in heterologen Expressionssystemen ein partieller Agonist ist.

Während ATP allein den $P2Y_{12}$ Rezeptor aktiviert, konkurrieren in Anwesenheit von ADP beide Substanzen um die Bindung am aktiven Zentrum und durch diesen partiellen Agonismus wird schließlich die Aktivität reduziert. Diese Ergebnisse veranlassen zu der Frage, ob ATP auch *in vivo* ein Agonist am $P2Y_{12}$ Rezeptor ist. Das ATP-ADP-Verhältnis der elektronendichten Granula der humanen Thrombozyten beträgt circa 2:1. Vorangegangene Studien zeigten, dass die Oberflächenkonzentration von ADP während der Thrombinstimmulation zwischenzeitlich 7–10 µM erreicht. Sowohl die ADP- als auch die ATP-Konzentration genügen, um den $P2Y_{12}$ der Thrombozyten zu aktivieren, vorausgesetzt deren Freisetzung aus den Granula geht mit derselben Kinetik einher. Es ist daher sehr wahrscheinlich, dass der $P2Y_{12}$ auch *in vivo* ein ATP-Rezeptor ist.

Das Anfügen einer 2'-methylthio-Gruppe an ATP verstärkte die Potenz am $P2Y_{12}$ und führte zu einem hoch potenten und vollen Agonisten. Die

Annahme, dass alle ATP-Derivate wahrscheinlich eine identische Bindungstasche besitzen, konnte mittels Ligandendocking im komparativen Strukturmodel des $P2Y_{12}$ verstärkt werden. Auch wenn dieses Modell derzeit nicht in der Lage ist vorherzusagen, welche Voraussetzungen für ein Nukleotid nötig sind, um agonistische oder inverse agonistische Eigenschaften zu erlangen, zeigt es, welche Aminosäureseitenketten für die Ligandeninteraktion unabdingbar sind. Bei 6 der an der Bindungstasche partizipierenden 7 Aminosäuren H^{253}, I^{257}, Y^{259}, T^{260}, Q^{263}, T^{264} und K^{280} ergab sich, dass an den entsprechenden Positionen jede Mutation zu einem Funktionsverlust des Rezeptors führt. Eine klare Übereinstimmung von experimentellen und Dockingstudien-Daten bestätigte dies.

Viele GPCRs, wie Histamin-, Thyrotropin- und Melanocortinrezeptoren, weisen eine hohe Basalaktivität auf. Im Unterschied zu Antagonisten können inverse Agonisten die agonistenabhängige und -unabhängige Aktivität des Rezeptors herabsetzen. Dies stellt besonders in der prophylaktischen Therapie einen großen Vorteil dar. Die derzeit therapeutisch genutzten $P2Y_{12}$-Liganden sind hoch affine Antagonisten. Inverse Aktivität wurde nur für den experimentellen $P2Y_{12}$ Blocker AR-C78511 beschrieben. AR-C78511 ist ein 2-alkylthio-substituiertes ATP-Analogon, welches im Unterschied zu mant-dATP keine Modifizierung an der 2’ oder 3’ OH Gruppe der Ribose aufweist. In dieser Studie konnten mant-dATP und mant-dADP als inverse Agonisten am $P2Y_{12}$ sowie verschiedene konstitutiv aktive $P2Y_{12}$-Mutanten identifiziert werden. Bei mant-dATP handelt es sich am wahrscheinlichsten um einen orthosterischen Liganden, bei dem die invers agonistische Aktivität an die Desoxyribose geknüpft ist, da mant-ATP die invers agonistische Aktivität verliert. Trotz der Identifizierung eines inversen Agonisten, ist es derzeit auch mittels strukturbasiertem Homologie-Modelling von GPCRs und qualifizierten Docking-Algorithmen nicht möglich, agonistische und invers agonistische Aktivität zu erklären, oder für Liganden vorherzusagen.

Anhang

(Supplemental Material)

Supplementary Information – Molecular Pharmacology

Identification of determinants required for agonistic and inverse agonistic ligand properties at the ADP receptor $P2Y_{12}$

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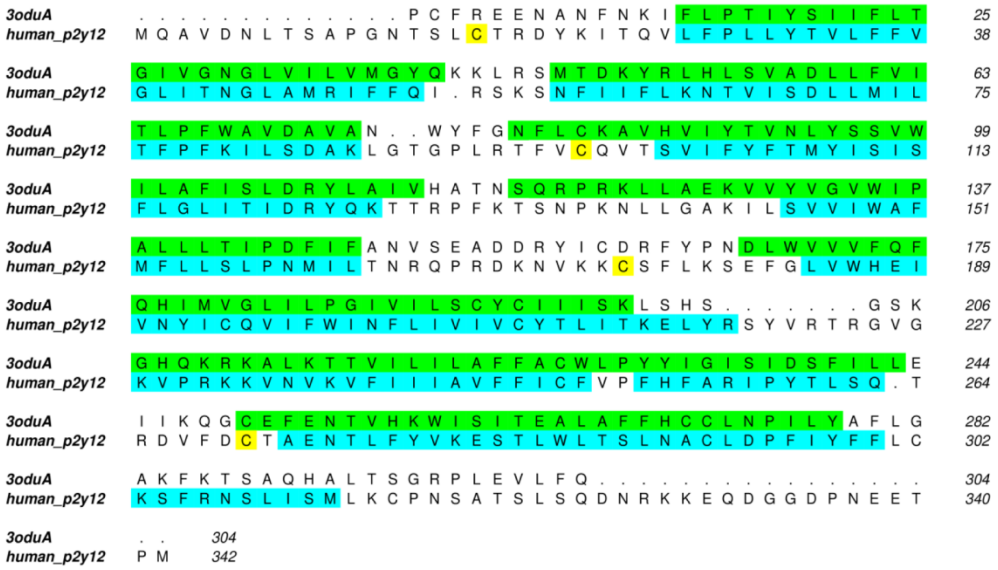
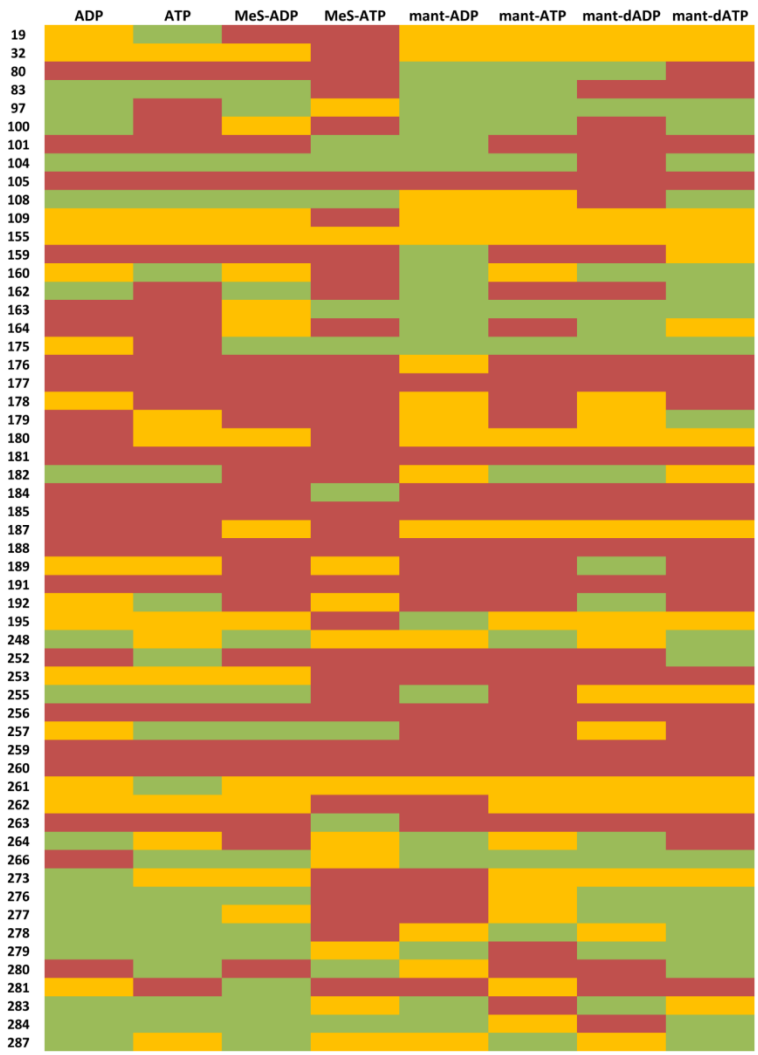
Figure S1



Suppl. Figure S1. **Sequence alignment of $P2Y_{12}$ with CXCR4.**

The sequence of human $P2Y_{12}$ was aligned with the sequence of human CXCR4 (PDB ID: 3ODU) (Gupta et al., 2001) using CLUSTALW (Larkin et al., 2007). Transmembrane helical regions of the CXCR4 receptor are highlighted in green. Regions predicted to be transmembrane helical regions of $P2Y_{12}$ according to secondary structure prediction server PSIPRED (McGuffin et al., 2000) are highlighted in cyan. Cysteine residues known to form disulphide bonds are highlighted in yellow.

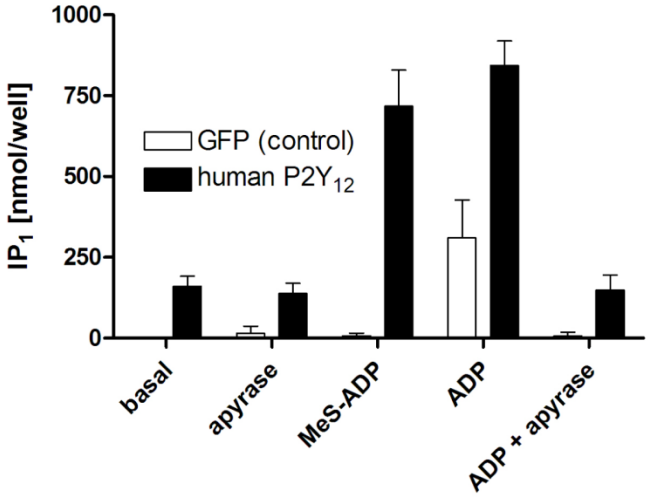
Figure S2



Suppl. Figure S2. **Per residue change in free energy with and without ATP derivatives bound.**

Rosetta was used to determine the difference in change in free energy (ΔΔG) between $P2Y_{12}$ with and without ATP derivatives bound. Residues that demonstrated the largest difference in free energy change in respect to bound ADP, ATP, MeS-ADP, MeS-ATP, mant-ATP, mant-ADP, mant-dADP and mant-dATP are listed. For each ligand, residues with ΔΔG above the average energy change (-0.24) are in red, while those with energy change above average but still negative are in yellow and residues with a positive energy change are in green.

Figure S3



Suppl. Figure S3. **Analysis of the functional impact of endogenous nucleotide released from CHO cells**

To analyze of whether endogenous nucleotide released from CHO cells contribute to basal activity of $P2Y_{12}$ we performed control experiments with CHO-K1 cells stably transfected with $G\alpha_{qi4}$. Intracellular inositol phosphate (IP_1) levels were determined with an immunological assay (cisbio Bioassays, IP-One ELISA, part-no. 72IP1PEA).

$G\alpha_{qi4}$-CHO-K1 cells transiently transfected with $P2Y_{12}$ presented an increased basal IP_1 level compared to cells transfected with the control plasmid (GFP). Incubation with 12.5 U/ml apyrase did not reduce this elevated IP_1 level. This clearly indicates that $P2Y_{12}$ does induce signal transduction by intrinsic active receptor conformation and not by nucleotides released from the cells into the medium. Proper $P2Y_{12}$ transfection was control by application of 100 µM ADP and 100 µM MeS-ADP. Proper apyrase function was demonstrated by loss of ADP action on $P2Y_{12}$.

Table

Suppl. Table S1. **Purine compound library screening at the human WT $P2Y_{12}$**

The activity of compounds (given in %) at the human $P2Y_{12}$ expressed in yeast is shown relative to the basal activity ($OD_{600\ nm}$ =0.089; set 0%) and the stimulation with 10 µM MeS-ADP ($OD_{600\ nm}$ =0.667; set 100%). Stimulation was measured after 24 h with 10 µM of the respective compound.

AMP -2.19	ADP 84.05	ATP 19.93	AP4 -0.29	cAMP -0.99
3'-dATP -2.02	7-Deaza-dAMP -1.33	NPE-caged-ATP -0.64	7-Deaza-dATP -1.33	DMB-caged-ATP 5.24
dATPαS -1.68	ADPβS 92	ATPγS 71.09	β-Methylene-APS -1.68	ApCp -0.81
AP3A -0.99	AP4A -1.5	AP5A -1.68	AP6A -1.68	AP4U -0.99
AP4(8I)G -2.37	AP5(8I)G -1.68	2'I-AMP -1.33	2'I-ADP -1.68	2'I-ATP -1.16
8Br-ADP 0.57	8Br-ATP -1.68	8Br-dATP -0.99	γ-[6-Aminohexyl]-ATP -1.5	2I-ATPγS 90.1
7-Deaza-7Br-dATP -2.02	γ-Aminophenyl-ATP 2.99	γ-[(6-Aminohexyl)-imido]-ATP -1.16	γ-[(8-Aminooctyl)-imido]-ATP -1.16	N6-(4-Amino)butyl-ATP -1.33
EDA-ATP -3.75	γ-[6-Aminohexyl]-N6-Benzyl-ATP -2.02	2-Hydroxy-ATP -1.5	TNP-ADP 72.81	TNP-ATP 4.03
1-Methyl-AMP -0.99	1-Methyl-ADP 1.61	1-Methyl-ATP 0.05	dATP 0.57	ddATP 0.57
2'.5'-pAp -0.47	ara-ATP 0.4	AMPαS -0.12	ATPαS 2.3	dADPαS 0.4
ApCpp -1.33	AppCp 0.4	dApCpp -0.47	AppNp 0.57	AppNH2 76.79
AP5U -1.16	AP4T -0.29	AP5T -0.47	AP4G 0.22	AP5G 0.05
2'Br-ADP -1.16	2'Br-ATP -0.64	2'-Ome-ATP -0.81	mante-ATPγS -0.4	8Br-cAMP 0.05
2'F-AMP -0.81	2'F-ATP -0.47	2'Cl-ATP -0.81	BzBzATP -0.12	7-Deaza-7I-dATP -0.12
N6-(6-Amino)hexyl-ATP -0.81	N6-(6-Amino)hexyl-dATP -0.12	8-[(4-Amino)butyl]-amino-ATP -0.12	8-[(6-Amino)hexyl]-amino-ATP 0.57	EDA-ADP 1.95
mant-ADP 6.79	mant-ATP -0.47	mant-dATP -0.64	mant-N6-Methyl-ATP -0.29	ε-ATP 0.4
Adenine 0.6	Adenosine 0.75	IMP 0.15	UDP 2.54	Xanthine 0.75
GDP 1.94	GTP 0.75	GTPγS 0.45		

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Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar, noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren.

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Publikationen und Vorträge

Publikationsverzeichnis

Schmidt, P., Ritscher, L., Dong, E.N., Hermsdorf, T., Cöster, M., Wittkopf, D., Meiler, J. and Schöneberg, T. (2013) Identification of determinants required for agonistic and inverse agonistic ligand properties at the ADP receptor $P2Y_{12}$. Mol Pharmacol. 83, 256-66

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Vorträge und Poster im Rahmen der Promotion

Summer school 2011 des SFB 610 in Dessau: "Identification of an inverse Agonist at the ADP receptor $P2Y_{12}$"

4^{th} Joint German-Italien Purine Club Meeting, Bonn 2011: "Identification of an inverse Agonist at the ADP receptor $P2Y_{12}$"

Danksagung

Ich möchte mich an dieser Stelle bei all Jenen bedanken, die mich im Laufe dieser Arbeit durch ihre Mitwirkung unterstützt und gefördert haben.

Mein Dank gilt hier an erster Stelle Prof. Dr. Torsten Schöneberg für die Organisation, Entwicklung und das Anvertrauen des interessanten Themas. Nur durch die kontinuierliche Betreuung und unermüdliche Unterstützung war eine Publikation möglich.
Außerdem gilt mein Dank allen Mitarbeitern der AG Schöneberg für ihre kollegiale Arbeit und die kreative und kritische Diskussion von Ideen und Problemen. Im Laboralltag konnte ich mich nicht nur auf methodische Unterstützung, sondern auch auf die zeitweise nötigen Aufmunterungen und motivierenden Worte verlassen.
Für finanzielle Unterstützung danke ich der Deutschen Forschungsgemeinschaft und dem SFB610.

Mein persönlich tiefster Dank gilt dennoch meiner Familie, auf die ich mich stets verlassen kann.

Ohne euch hätte ich es nie geschafft. Vielen Dank.